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The associations between nutrient intake and eating patterns with adiposity, metabolic risk factors, and the gut microbiome in Hispanic college freshmen

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The associations between nutrient intake and eating patterns with adiposity, metabolic risk factors, and the gut microbiome in Hispanic college freshmen

by

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Dedication

This work is dedicated to:
My mother, Yun-Chu Pilles, so she knows her life sacrifices were worth every setback.
My grandmother, Sun-Cha Buckner, so she knows the things she risked in coming to this country afforded me this opportunity.
My father, Michael Pilles, so he knows his silent support actually gave me strength.
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The associations between nutrient intake and eating patterns with adiposity, metabolic risk factors, and the gut microbiome in Hispanic college freshmen

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Hispanics are at an increased risk of obesity and metabolic disease in the United States. Some studies have shown 70% of college students gain weight during their freshman year and is a critical transition period contributing to the rise in obesity rates. Research indicates that obesity is associated with an altered gut microbiome, a dynamic community of microbes that encode for proteins that perform diverse metabolic roles, are associated with disease states and may be affected by dietary intake. The objective of this study was to examine the relationship between the gut microbiome and dietary intake, eating patterns, adiposity, and metabolic measures in a population of college Hispanic freshmen. This cross-sectional study and secondary analysis was performed at The University of Texas at Austin and involved Hispanic college freshmen (18-19 years of age). The first aim was to examine the relationship between dietary intake, adiposity, and metabolic markers. Fiber intake was negatively associated with hepatic fat (HF), glucose, insulin, and leptin. Saturated fat intake was positively associated with HF, subcutaneous adipose tissue (SAT), total body fat, cholesterol, insulin, insulin resistance, leptin, and C-reactive protein (CRP). Saturated fat intake was also associated with increased odds of non-alcoholic fatty liver disease (NAFLD). The second aim was to examine the relationship between the gut microbiome and the diet. Subjects who met saturated fat

recommendations compared to those who exceeded recommendations had a more diverse microbiome. The third aim was to evaluate the relationship between the gut microbiome, adiposity, and metabolic measures. Subjects who had a low percent body fat, low insulin values, and high LDL values had a more diverse microbiome compared to subjects with a high percent body fat, high insulin values, and low LDL values. Collectively, these data suggest that an intake of saturated fat is associated with unfavorable health outcomes and the microbiome may be a possible mechanism and/or target for treatment and prevention.

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List of Abbreviations

BF- Burkino Faso
BMI- Body Mass Index
BMI-z- Body Mass Index z-score
CAFE- controlled-feeding experiment
CONV-R- Conventionally-raised mice
CRP- C-reactive Protein
DRI- Dietary Reference Intakes
EO- Eating Occasion
EU- Italy
FDR- False Discovery Rate
FFQ- Food frequency questionnaire
GF- Germ-free
GI- Gastrointestinal
GIP- Glucose-dependent Insulinotropic Polypeptide
GLP-2- Pro-glucagon derived Peptide
HDL- High Density Lipoprotein
HF- Hepatic Fat
HMP- Human Microbiome Project
HOMA-IR- Homeostatic Model Assessment- Insulin Resistance
IHTG- Intrahepatic triglycerides
IL-6- Interleukin-6
IL-10- Interleukin-10
LDL- Low Density Lipoprotein
MANCOVA- Multiple Analysis of Covariance
MetaHIT- Metagenomics of the Human Intestinal Tract
MRI- Magnetic Resonance Imaging
NAFLD- non-alcoholic fatty liver disease
NDSR- Nutrition Data System for Research
OTU- Operational Taxonomic Units
PCR- Polymerase Chain Reaction
QIIME- Quantitative Insights Into Microbial Ecology
SAT- Subcutaneous Adipose Tissue
SCFA- Short-chain fatty acid
SSB- Sugar sweetened beverages
T2D- Type 2 diabetes
TNF- α- Tumor Necrosis Factor alpha
UAB- The University of Alabama at Birmingham
US- United States
UT- Austin- The University of Texas at Austin
VAT- Visceral adipose tissue
WC- Waist Circumference

Chapter1: Introduction

Hispanics are one of the largest and fastest growing ethnic minorities in the United States and in recent history have surpassed Non-Hispanic Whites and Blacks in college enrollment.[1,2] In 2013, Hispanic students represented 23% of freshman enrollment at the University of Texas at Austin (UT-Austin), having the largest increase among all minority groups.[3] However, the freshman year of college is known to be a transitional period into autonomy and young adults are able to choose their own dietary eating habits. In the United States (US), college students consume more junk food and alcohol, and less dietary fiber, fruits and vegetables.[4–6] Several studies have shown that 70% of college freshmen gain an average of 3.5 to 7.7 pounds in the first year of college[4,7–9] with no difference in dietary intake and physical activity from their freshman year to their senior year.[4,10] In addition, Hispanics are also disproportionately affected by obesity, type-2 diabetes (T2D), and non-alcoholic fatty liver disease (NAFLD).[11] Therefore, the transition to college is as a critical period contributing to the rise in obesity rates, as the behavioral choices college students may likely affect their risk of chronic disease later in life, specifically in a high-risk Hispanic population.

Studies done on an exclusive Hispanic youth or young adult population have found that diets high in added sugar and low in dietary fiber as well as decreased eating frequency and skipping breakfast have been positively linked to obesity levels, visceral adipose tissue, insulin resistance, and circulating lipids in Hispanic youth and young adults.[12–17] The gut microbiome may be a possible mechanism linking diet and eating behaviors with differences in adiposity and metabolic measures.

The human gut is a host to a diverse and dynamic community of microbes that encode proteins not independently present in the human genome and that play numerous diverse roles in metabolism and energy homeostasis. The structure and composition of the gut microbiota is hypothesized to reflect host natural selection by promoting specific bacteria that acts in stabilizing and diversifying the human gastrointestinal (GI) tract whose collective behavior is beneficial to the host.[18] The five major bacterial phyla that dominate the gut are *Firmicutes, Bacteroidetes, Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. Current research suggests that disruptions in the normal balance of gut microbial populations, specifically decreases in microbial diversity, are linked to a variety of gut-related disease and conditions, including obesity, inflammatory bowel disease, T2D, and colorectal cancer.[18–24] Although what constitutes a "healthy" gut microbiome remains unknown, it is clear that diversity and redundancy of microbial populations is essential.

Some studies have assessed the influence of diet on microbiome composition. A study comparing children in Burkina Faso (BF) with children in Italy (EU) found significant increases in *Bacteriodetes* and microbiome diversity in Burkina Faso (BF) children, who consumed significantly less fat, sugar, starch, and animal protein, and significantly more fiber. The BF children also had significantly more short-chain fatty acid (SCFA)-producing bacteria that aid in prevention of pathogenic bacteria while the EU children had significantly more pathogenic bacteria such as *Shigella* and Salmonella.[25] A 10-day randomized hospital controlled-feeding experiment in adult humans (CAFE; n=10)[26] and humanized mice[27] showed significant changes in individual microbiome composition within 24 hours of switching to a high-fat/low-fiber (Western

diet) or a low-fat/high fiber diet, but the magnitude of changes were modest over time. A study comparing conventionally raised mice (CONV-R) and germ-free (GF) mice showed that CONV-R animals had 40% more total body fat than their GF counterparts fed the same polysaccharide-rich diet, even though CONV-R animals consume less chow per day.[28] When CONV-R cecal content was then donated to the GF mice, the body fat of the GF mice increased to the levels of CONV-R mice. Collectively, these studies demonstrate that the microbiota is associated with and can be immediately altered by dietary intake. In addition, although caloric density of food items are portrayed as a fixed value on package labels, it seems caloric value varies between individuals according to their intestinal microbiota composition. A highly efficient microbiota would promote energy-harvesting and storage (obesity) while a less efficient microbiota would promote leanness.[18]

Few studies have investigated the relationship between adiposity and cardiovascular risk factors with the microbiome. A study done in elderly adults demonstrated a weak association between microbiota and blood pressure.[29] A decrease in *Bacteriodetes* and increased *Firmicutes* and *Proteobacteria* were found in wild-type (obese) and knock-out (lean) mice when put on a high fat diet, independent of obesity.[30] Case-control diabetic studies found no significant difference in mean bacterial diversity between diabetic and nondiabetic subjects although significant differences were associated with specific bacterial phyla in the gut.[31,32]

Dietary intake plays a role in the microbiome but the mechanism and magnitude of influence is unknown. Significant and meaningful changes in the gut microbiota have been associated with dietary alterations, primarily consumption of dietary fiber, fat,

sugar, and being breastfed during infancy.[21,25,26,29] However, the above studies used food frequency questionnaires and screeners to collect information on dietary intake, which are limited in their ability to capture dietary patterns, energy intake and macronutrients. In addition, no study has examined the influence of eating patterns on the microbiome.

The largest study to date is being collected by the Human Microbiome Project Consortium and has a sample of 242 volunteers ranging in age from 18-40 of varying ethnicities with the goal of phenotyping a "healthy" human microbiome.[19,20] No study has examined the relationship between the microbiome and diet exclusively in a high-risk Hispanic young adult (18-19 years of age) population. Given this population is disproportionately affected by obesity and metabolic disease and college years are a critical transition period in which lifetime eating habits are established, understanding how diet impacts the gut microbiome in this population is warranted. Thus, the **overall goal** of this study is to examine the relationship between diet, adiposity and metabolic measures, and the gut microbiome in Hispanic college freshmen.

Chapter 2: Literature Review

DISEASE IN HISPANIC POPULATIONS

Growing Hispanic population

As of 2014-2015, there were 56.6 million people of Hispanic origin or 17.6% of the total population in the US, making up the largest ethnic minority[33]. Hispanics are one of the fastest growing ethnic minorities in the US and are projected to make up 28.6% of the nation by 2060.[33] Specifically in Texas, Hispanics comprise over one third or 39% of the state population with 87% being of Mexican origin.[34] This Hispanic population growth is also reflected in US college enrollment.

In 2012 Hispanics surpassed Non-Hispanic Whites and Blacks in college enrollment immediately following high school.[1] At UT-Austin, as of 2015, Hispanic students represented 23% of freshman enrollment.[3] Due to the increases in college enrollment and marked decreases in health during the freshman year of college, this population is at an increased risk of developing unhealthy lifestyle patterns that contribute to the increasing obesity and metabolic disease risk in the Hispanic population. The gut microbiome may be a possible mechanism linking diet and eating behaviors with increases in adiposity and disease.

Hispanic disease risk

Obesity affects one third of the US population and is associated with heart disease, cancer, and T2D, amongst other comorbidities that lead to preventable deaths.[35] This disease does not only hurt physically, but also hurts financially. The medical costs of an obese person are $1,429/year higher than that of a normal weight person.[35] There is no doubt that obesity is a burden on the US population. However, obesity affects some subpopulations more than others. Hispanic youth have the highest rates of obesity

compared to other ethnicities. Nearly one fourth of Hispanic children and adolescents and nearly half of Hispanic adults are classified as obese.[8] Obesity in US youth (2-19 years of age) has significantly increased from 13.9% in 1999 to 17.2% in 2014, and this trend continues to increase. This is particularly troubling because the rise in obesity means a rise in associated comorbidities such as NAFLD.

NAFLD is a liver condition of abnormal lipid deposition in the absence of excess alcohol intake and often accompanies obesity. NAFLD used to be diagnosed with a biopsy and was usually only discovered in someone exhibiting symptoms of NAFLD. With the development of non-invasive MRI techniques, it is now defined as a person who has more than 5.56% of liver fat for total liver volume in the general population.[36–39] Twenty four percent of obese Hispanic youth have NAFLD, and the prevalence of NAFLD is highest in Hispanics compared to Non-Hispanic Blacks and Non-Hispanic Whites.[36,40] An age-adjusted model for NAFLD showed that Mexican Americans are 69% more likely to be diagnosed with NAFLD compared to their Non-Hispanic White counterparts.[45] More than 4000 Hispanic youths (<20 years of age) are being diagnosed with NAFLD annually.[50] NAFLD can become inflamed and progress to nonalcoholic steatohepatitis (NASH) and eventually become cirrhosis.[42] Understanding and monitoring this population is paramount for reducing, preventing, and treating obesity and NAFLD[36,43] since NAFLD is the most common cause of liver disease and liver failure.[36,43]

Diabetes is a disease in which the body is unable to regulate insulin levels through various mechanisms.[44] This deregulation leads to an increase in blood glucose levels, which over time can cause heart disease, vision loss, nerve damage, and may lead to death.[44] Type 2 diabetes (T2D) often can be prevented with a healthy lifestyle and decreased adiposity stores like visceral adipose tissue (VAT), which is why T2D is often linked to obesity.[44] Like obesity, T2D affects subpopulations at higher levels. The prevalence of diabetes in Hispanics are 1.9 times higher than Non-Hispanic Whites, even after age-adjustment, and Hispanics are 50% more likely to die from diabetes compared

to Whites.[44,45] Specifically in Hispanic youth 15-19 years of age, incidence of T2D exceeded T1D, and occurred significantly more in obese teens.[41] This population being at an increased risk for developing T2D makes monitoring insulin and glucose levels of utmost importance.

Heart disease is the number one killer for Americans, and the second leading cause of mortality for Hispanics.[46,47] Although Hispanics have a lower incidence of heart failure than non-Hispanics, Hispanics have higher risks for cardiovascular risk factors due to the increased incidence of obesity and diabetes.[46,47] The connection between obesity, diabetes, and heart disease makes monitoring blood lipids and VAT deposits is essential.

THE RELATIONSHIP BETWEEN DIET AND DISEASE IN A HISPANIC POPULATION

Diet and obesity

Studies on diet and eating patterns in exclusive overweight Hispanic youth and young adult populations have found a variety of nutrients, food and beverages to be linked with obesity and metabolic diseases. A cross-sectional study in 175 Hispanic youth (8-18 years of age) found participants who consumed the most nutrient rich vegetables had 44% less liver fat and 17% less visceral adipose tissue (VAT) than those who consumed the least.[48,49] In a cohort of 120 Hispanic youth (10-17 years of age), increased total dietary sugar intake was associated with increased adiposity.[12] In the same cohort, breakfast skipping and decreased eating frequency (<3 eating occasions/day) was associated with increased obesity and VAT.[15,17] In a cross-sectional analysis of 60 overweight and obese Non-Hispanic Black and Hispanic adolescents (14-18 years of age), high intakes of sugar sweetened beverages (SSB) was associated with a 7% increase in VAT compared to low intakes of SSB.[50] In a sample of 187 Hispanic children (10-14

years of age), SSB was positively associated with BMI z-score.[51] These findings consistently show that while dietary fiber is inversely linked, added sugar intake, specifically SSBs, are positively linked to adiposity depots in Hispanic youth.

Diet and NAFLD

Reducing caloric intake and dietary weight loss has been a common and effective therapy for the treatment of NAFLD.[52] However, it is still unclear as to whether the weight loss and treatment of obesity is leading to a reduction in hepatocytes or whether the negative caloric balance or actual diet is driving the mechanism of this benefit. Increased protein intake was associated with a decrease in intrahepatic triglycerides (IHTG),[53] but meat intake was associated with NAFLD in another study.[54] Simple carbohydrates are associated with NAFLD, in particular high fructose corn syrup found in sugar sweetened beverages.[54,55] An intervention study with 24 overweight Hispanic adolescents (11-18 years of age) who had NAFLD and were regular consumers of SSBs were switched from fructose sweetened beverages to glucose sweetened beverages and saw an improvement in insulin sensitivity and LDL oxidation.[56] Total dietary fat as well as specific types of fat have been positively associated with NAFLD, however many studies did not account for overfeeding.[52] A study done on diet and NAFLD of 375 Israelis found no significance in dietary fat intake between normal and NAFLD subjects, after controlling for sex and energy intake.[54] The research on diet and NAFLD is unclear and this relationship warrants further research, particularly in Hispanic youth.

Diet and metabolic diseases

A healthy lifestyle including diet and exercise has been shown to prevent T2D and cardiometabolic diseases.[44] In Hispanic youth, the link between diet, T2D, and cardiometabolic diseases are minimal since these diseases evolve over time and are

usually diagnosed in adulthood. More studies have examined diet on diabetes and cardiovascular risk factors such as insulin resistance/insulin sensitivity and blood lipids. A cross-sectional study in 175 Hispanic youth (8-18 years of age) found participants who consumed the most nutrient rich vegetables (1.7 ±1.0 servings/day) compared those who consumed the least (0.1 ±0.1 servings/day) were more insulin sensitive.[49] In a cross-sectional study of 187 Hispanic children (10-14 years of age), SSB was positively associated with insulin resistance.[51] In another cross-sectional study of 120 Hispanic youth (10-17 years of age), increased eating frequency (>3 eating occasions/day) was associated with 29% lower fasting insulin, 31% lower insulin resistance, and 19% lower triglyceride count compared to youth who eat infrequently (<3 eating occasions/day).[16] A decreased eating frequency, increased intake of SSB, and decreased vegetable intake have been associated with T2D and cardiometabolic risk factors in overweight Hispanic youth. These dietary patterns may be exacerbated in a Hispanic emerging adult population.

Diet during the transition to college

"Emerging adulthood" is defined as ages 18-25 and is only present in societies that allow a time period for self-exploration.[57] This time period is hallmarked by behavior changes allowing a person to express his or her individuality, including establishing his or her own health patterns. Many longitudinal studies have shown an increase in obesity prevalence in emerging adults and the largest weight gains were seen during young adulthood.[4] In the first semester of college, students are likely to gain an average of 3.5 to 7.7 pounds.[7–9] This weight gain is likely to be sustained through adulthood as no difference in physical activity or eating habits were seen between lower-level and upper-level undergraduates and this trend continues into adulthood.[4,10,58,59] In addition, college students and emerging adults are more likely to eat fast food and alcohol, and less dietary

fiber, fruits and vegetables.[4–6,60] Therefore, the freshmen year in college is an important developmental stage to target in promoting and adopting long-lasting health behaviors.

WHAT IS THE GUT MICROBIOME?

The gut microbiome is an ecosystem of up to 100 trillion bacteria residing in the digestive tract.[61] Although bacteria exist in many human body environments, from the mouth to the anus, the distal intestine or colon is the most stable of the habitats and harbors most of the gut microorganisms.[18,61] The human gut microbiome has been described as an organ within an organ that consumes, stores, and redistributes energy and mediates certain chemical transformations.[18] As humans evolved from Neolithic hunter-gatherers to agricultural foragers to present-day industrialists,[18,61,62] it is hypothesized that the human gut microbiome and its nutrient harvesting capabilities had to adapt quicker than the genome to the ever changing environments and food sources.[18,61,62] Factors that influence the microbiome start in utero and fluctuate based on delivery method, infant feeding method, age, geography, pharmaceuticals, diet, and psychological and physical stress.[61] The gut microbiome possessing independent genes from the host genome, an ability to self repair and replicate, and change with age, provide evidence to its separate and rapidly evolving nature.[18,29] Natural selection is theorized to determine the composition of the gut microbiome to achieve optimal functionality; meaning the collective behavior of bacteria is beneficial to the host if the host provides the microbiota with beneficial nutrients.[18,23] However, the many variables contributing to the composition of the gut microbiome make this topic a difficult one to study.

Bacteria are classified in phylogenetic hierarchies, the largest and most encompassing being phylum followed by class, order, family, genus, and species; each one more specific than the previous. While species is the most specific of the classifications, it is also the least studied since historically microbes had to be grown *in*

situ in order to be analyzed.[63] Only recently did the current technology of 16S RNA sequencing with accompanying software such as QIIME and QWRAP allow relatively cheap and fast analysis to be done on *in vivo* samples.[64] Therefore, many studies have characterized their samples at the phylum level, but not always the species level because many named species are not available, having yet to be defined or discovered.

The Human Microbiome Project (HMP) set out to phenotype the "healthy microbiome" by collecting 4,788 specimens from different body habitats, in 242 screened healthy adults of varying ages and ethnicities in the United States. No taxa were observed to be universally present in all habitats and no two healthy individuals had the same microbiome.[19,61] A similar study, Metagenomics of the Human Intestinal Tract (MetaHIT), catalogued and phenotyped 124 subjects in Spain and Denmark and also found there were marked differences between individuals.[65] In a healthy subsample of both studies, *Bacteroidetes* and *Firmicutes* were found to be the most abundant phyla in the healthy gut, but varied widely in proportion. HMP displayed 75.7% relative abundance of *Bacteroidetes* and 20.5% *Firmicutes*, while MetaHit displayed 45.8% *Bacteroidetes* and 46.8% *Firmicutes*. Therefore, a "healthy microbiome" remains to be defined. Many studies have set out to characterize the differences between a diseased gut and a healthy one.

To measure the difference between two individuals or two populations, the relative abundance and diversity of gut bacteria are compared. Abundance measures the count of similar bacteria and diversity is the number of different species within the gut.[64,66] A dysbiotic gut is characterized as having an overall decreased abundance and diversity. Dysbiosis can occur due to diet, drugs, or disease and results in an altered microbiome that promotes disease in the host.[23] Substantial differences have been seen in many gut-related conditions such as inflammatory bowel disease, NAFLD, obesity, and diabetes, amongst others.[32,42,43,67–70]

DOES DIET DRIVE THE MICROBIOME?

Diet is the energy source of the gut microbiome.[18] However, scientists were not sure if the microbiome determined what humans consumed or if the food source determined microbiome composition. A study investigating healthy children (1-6 years of age) in rural Burkino Faso (BF) and Westernized Europe (EU) found children under the age of two still being breast-fed had similar microbiome environments in both populations, despite differences in geography, genetics, and sterilization.[25] This indicated that food sources were driving factors of the microbiome. In addition, BF children over the age of two consumed significantly more fiber and less total energy, fat, protein, and carbohydrates per day than their EU counterparts. This resulted in significantly more relative abundance of *Bacteroidetes* and lower counts of pathogenic bacteria. Studies directly measuring the differences in the human gut microbiome between omnivores, vegetarians, and vegans found no overall significance between the diets.[71,72] However, vegetarians did have significantly lower microbial counts of *Bacteroides* and *Bifidobacterium* compared to omnivores, and vegans had significantly lower *Bacteroides*, *Bifidobacterium*, *E.coli*, and *Enterobacteriaceae* compared to omnivores.[72] Stool pH was analyzed and vegans had the lowest pH. This alludes to a possible mechanism between pathogenic bacteria like *E.coli*, and *Enterobacteriaceae* and their inability to survive in acidic environments. Collectively, these studies show that diet is an obvious factor influencing bacteria and that high fiber, plant-based diets cultivate an advantageous environment.

Intervention studies have tried to discover if changing the diet can change the composition of the microbiome. Animal studies have shown mice on a 13 week standard diet compared to mice on 21 week high fat diets, resulted in changes in microbiome composition, independent of changes in body weight.[30] Mice on a standard diet had significantly more *Bacteroidetes* and less *Firmicutes* than mice on a high fat diet. Mice

on a low fat, high protein diet that were switched to a Western, high fat, low protein diet showed an increase in body fat and *Bacteroidetes*. In humans, a short-term controlled-feeding experiment (CAFE) randomized ten subjects into high-fat/lower fiber or low-fat/high fiber diets over ten days. Changes in the microbiome were detected after twenty-four hours but were not lasting, and the direction of specific bacterial change was not stated.[26] Other interventions included type of breakfast consumption, in which 30 volunteers were given a whole grain oat breakfast or a non-whole grain breakfast.[73] The whole grain group saw an increase in relative abundance of *Bifidobaceria* and overall diversity, and a reduction in LDL cholesterol. In conclusion, these intervention studies indicated that the gut microbiome is malleable with diet, although the intervention must be sustained.

The majority of studies examining how diet relates to the gut microbiome have used food-frequency questionnaires (FFQ). In the HMP, increased intake of carbohydrates, as measured by FFQ, was correlated with *Methanobrevibacter*, *Prevotella*, and *Candida*.[74] Increased intake of animal protein was correlated with *Bacteriodes*. Saturated fatty acids were correlated with *Candida*, polyunsaturated fats were correlated with *Nitrososphaera*, and total fat intake was correlated with *Bacteriodes*. However, FFQ are limited in their ability to capture daily food intake patterns, rely heavily on long stretches of memory, and are closed ended questions. The diet in current population-wide human observational studies were evaluated using FFQs, none used 24-hour diet recalls.

MICROBIOME, OBESITY, AND METABOLIC HEALTH

Several studies have provided evidence of the gut microbiota regulating fat storage. The first studies were performed in mouse models between conventionally-raised (CONV-R) mice and germ-free (GF), microbiome knock out mice.[28] Although germ-free

mice consumed 29% more chow, the CONV-R mice had 42% more total body fat than the GF mice. In addition, when the GF mice received a microbiota cecal transfer from the CONV-R mice, total body fat in the previous GF mice matched that of their donor. This study revealed the capacity of the gut microbiome to harvest and store energy and showed the microbiome directly and immediately affects host weight gain and adiposity storage. Another study relating to adiposity was done in 32 monozygotic and 23 dizygotic twin pairs (21-32 years of age) to measure abundance and diversity of humans who were genetically similar.[75] Obese twins had significantly less abundance and diversity than lean twins, regardless of genetic similarity. In addition, a decrease in diversity was associated with an increased abundance of *Actinobacteria* and a decrease in *Bacteroidetes*.[75] This suggests that the microbiome does not adapt to energy, but reduces replication and diversification in an abundance of energy input.[75]

In humans, specific bacteria at the phylum and class level have been associated with NAFLD, diabetes, and cardiometabolic diseases. A cross-sectional study of healthy, NAFLD, and NASH patients did not find overall significance between groups, but found a significantly lower relative abundance of *Bacteroidetes* in NASH patients compared to both NAFLD and healthy patients, even after controlling for BMI.[42] In 36 males, 18 of which had T2D, did not have a significance difference in overall diversity between diabetic and non-diabetic patients. However, decreases in phylum *Firmicutes* and class *Clostridia,* and increases in class *Betaproteobacteria* were observed in diabetic patients compared to health patients.[32] In 531 Finish men with metabolic syndrome, *Coriobacteriacea* was associated with impaired glucose tolerance. Together, these studies suggest the type of bacteria dominant in the gut determine which nutrients get broken down and stored, and may be the mechanism behind obesity and its comorbidities.

CONCLUSION

In conclusion, the gut microbiome is an emerging field where much more research is needed. Hispanics are at an increased risk of diseases that are influenced by poor dietary habits, and habits worsen with the transition to college, thereby increasing disease risk. Although current research has shown that the gut microbiome is directly altered by diet and disease, phenotyping an exclusive Hispanic population has not been done. The need to understand how diet impacts the gut microbiome and the possible mechanism by which the gut microbiome impacts metabolic outcomes is imperative.

Therefore, the overall goal is to examine the relationship between diet, adiposity, metabolic markers, and the gut microbiome in Hispanic college freshmen. **The aims of this project were as follows: 1**: To examine the relationship between dietary intake and adiposity depots, specifically visceral adipose tissue (VAT) and hepatic fat (HF), and metabolic parameters, specifically fasting glucose, insulin, lipids, and inflammatory adipokines. **2.** To examine the relationship between the gut microbiome, adiposity, and metabolic measures (specifically body fat and NAFLD). **3.** To examine the relationship between the gut microbiome, dietary intake (including macronutrients, fiber, and added sugar intake) and eating patterns (i.e. eating frequency and breakfast intake). The hypotheses were that diets high in sugar and saturated fat, and low in dietary fiber would be linked to poor adiposity and metabolic outcomes, and decreased microbial abundance and diversity.

Chapter3: The link between dietary intake and adiposity and metabolic parameters in Hispanic college students.

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ABSTRACT

Background: The transition to college is a critical period contributing to dietary choices that likely affect students' adult chronic disease risk. Hispanics are the fastest growing ethnic population enrolling in college today. However, Hispanics are disproportionately affected by T2D, obesity, and non-alcoholic fatty liver disease.

Objective: The purpose of this study is to examine the relationship between dietary intake, adiposity depots, and metabolic parameters in college Hispanic freshmen.

Design: A cross-sectional study of 98 Hispanic college freshmen (18-19 y) with the following measures collected: height, weight, waist circumference, body mass index via BodPod, hepatic fat (HF), visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT) via MRI, glucose, insulin, insulin resistance (HOMA-IR) and lipids via fasting blood draw, and dietary intake via multiple 24-hour dietary recalls. MANCOVA analyses were performed using adiposity and metabolic measures as dependent variables and dietary intake as independent variables, controlling for total energy intake and sex.

Results: Total dietary saturated fat intake was positively related to HF, SAT, total body fat, insulin, HOMA-IR, leptin, and total cholesterol. The odds of having non-alcoholic fatty liver disease was increased by 36% with every 1% increase in dietary saturated fat. Total fiber intake was inversely related to HF, glucose, HOMA-IR, and leptin. Total carbohydrate intake was inversely linked to CRP levels, while total sugar intake was related to higher triglycerides, and added sugar intake was related to higher VAT.

Conclusion: Our findings support previous findings that diets high in saturated fat and sugar intake contribute to increased adiposity and metabolic disease risk; while diets high in fiber intake contribute to lower adiposity and metabolic disease risk in Hispanic college students. This data will be useful to guide future obesity prevention and treatment programs in this population.

INTRODUCTION

College a transitional period of time when young adults in the United States (US) consume more junk food and alcohol and less dietary fiber, fruits and vegetables compared to their pre-college diets.[4–6] Several studies have shown that 70% of college freshmen gain an average of 3.5 to 7.7 pounds in the first year of college[4,7–9] with no improvements in their dietary intake and physical activity from their freshman year to their senior year.[4,10] Therefore, the transition to college has been identified as a critical period contributing to the rise in obesity rates as the negative behavioral choices college students make will likely affect their risk of chronic disease later in life.

Hispanics are the largest and fastest growing ethnic minority in the US and in recent years have surpassed Non-Hispanic Whites and Blacks in college enrollment.[1,2] In 2015, Hispanic students represented 22% of freshman enrollment at the University of Texas at Austin (UT-Austin), having the largest increase among all minority groups.[3] Hispanics are also disproportionately affected by obesity, type 2 diabetes (T2D), and non-alcoholic fatty liver disease (NAFLD).[11] Previous research has shown that diets high in added sugar and low in dietary fiber are positively linked to obesity levels, specifically visceral adipose tissue (VAT), insulin resistance, and circulating lipids in Hispanic youth and young adults.[12,13,15–17,76] Thus, the goal of this study was to examine the relationship

of dietary intake with adiposity and metabolic measures in an exclusive sample of Hispanic college freshmen to better inform dietary interventions that may reduce the risk of metabolic disease during this critical time period.

SUBJECTS AND METHODS

Participants

Figure 3.1 provides a detailed flow of study participants. The original purpose of this study was to examine the relationship between eating frequency and adiposity and metabolic markers. Hispanic college freshmen subjects were recruited via announcement in classes, word of mouth, electronic posted notices, and tabling at dorms around the UT-Austin campus and completed a screener to determine eligibility. Inclusion criteria included: (i) self-reported that all four of their grandparents were of Hispanic origin (ii) 18-19 years of age, and (iii) in their first year of college. Exclusion criteria included (i) if they were pregnant, (ii) if they were taking any medication known to affect body composition or any psychoactive medication, (iii) if they had been diagnosed with a disease/s or syndrome known to affect body composition or fat distribution, (iv) if they had a learning impairment that would complicate survey administration, (v) if they had braces, a pacemaker, or any other contraindications to magnetic resonance imaging (MRI) scanning, or (vi) if they had taken part in a weight loss, dietary, or physical intervention in the previous six months. Of the 791 eligible Hispanic students, diet recalls were conducted in 100 subjects. Two subjects were excluded from all of the analyses because a) one subject did not have any MRI or blood draw data, and b) another subject was an extreme outlier in percentage of caloric intake from carbohydrate due to

low carbohydrate intake (< 10 grams per day). Ninety-two subjects had complete adiposity and lipid measures, and 90 had complete glucose/insulin and adipokines measures.

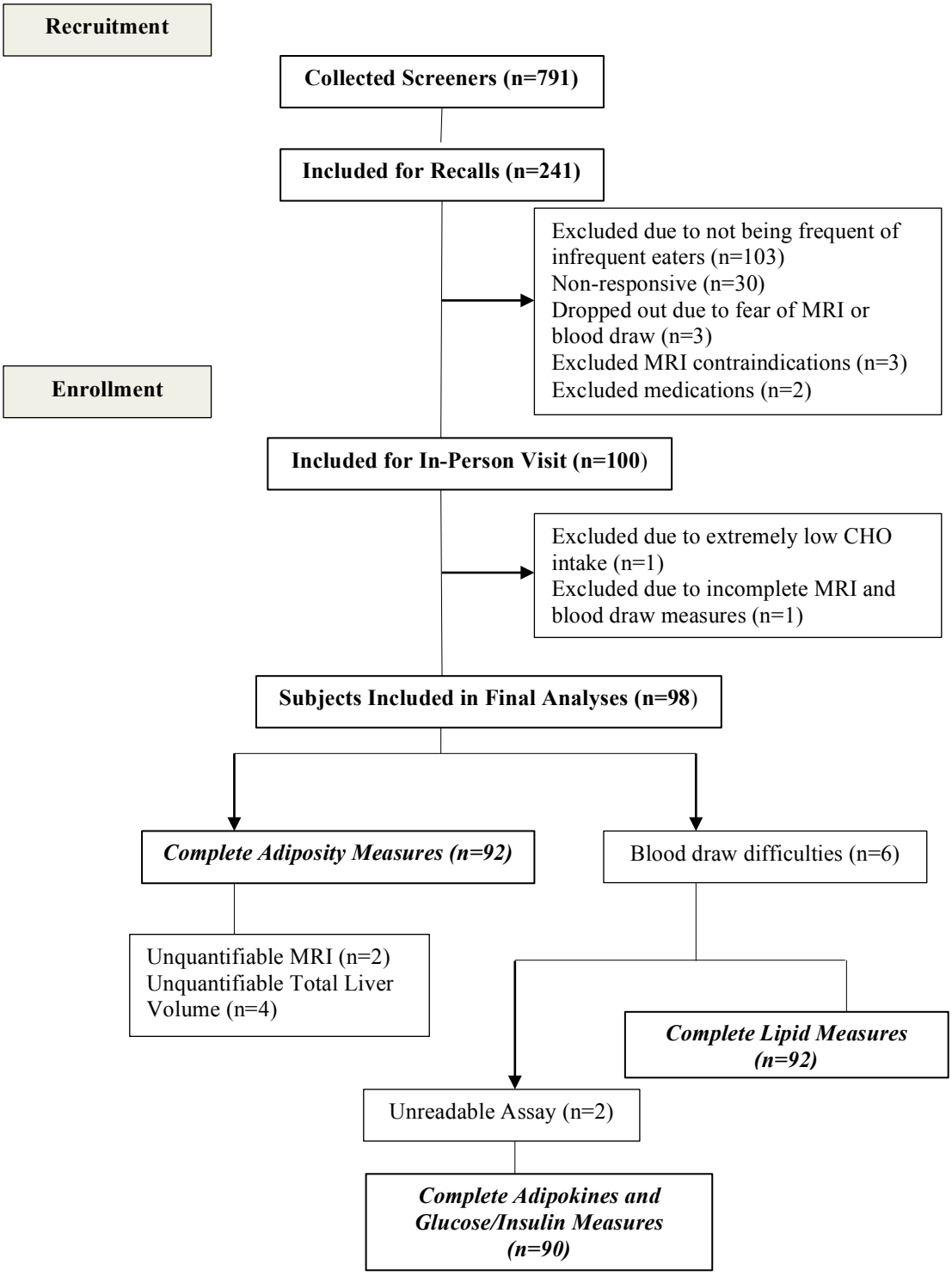

Figure 3.1: Flow of participants through the study.

Dietary Intakes

Dietary intakes were assessed from three to four 24-h diet recalls using the multiple-pass technique. Research staff collecting the diet recalls were trained and supervised by a Registered Dietitian. Subjects had at least three recalls (one weekend and two weekdays). On average recalls were administered within five days from the in-person testing visit. All recalls were then double checked by another trained research staff. Nutritional data was analyzed by using the Nutrition Data System for Research (NDS-R, 2014). The NDS-R program calculated key dietary variables for this analysis, including mean energy, total fat, protein, carbohydrates, saturated fat, total sugar, added sugar, dietary fiber, soluble fiber, and insoluble fiber. Prospectively, no recall was performed if the subject indicated being ill. Plausibility of energy intake was assessed by regressing caloric intake against BMI and there were not subjects that were over 2 standard deviations from the mean (n=98).

Anthropometrics and Adiposity Measures

Height and weight were measured to the nearest 0.1 kg and 0.1 cm using a beam medical scale and a wall-mounted stadiometer, respectively, and the average of two measurements was used for the analysis. BMI was calculated utilizing adult cut offs and body mass index (BMI) percentiles and z-scores (BMI-z) were determined by using EPII 2000 software (version 1.1; Centers for Disease Control and Prevention, Atlanta, GA). Subjects were categorized as overweight if they had a BMI of 25.0 to < 30.0 and obese if they had a BMI >30.0. Waist circumferences (WC) were measured and recorded to the nearest 0.1 cm. Body fat and soft lean tissue were measured using the BodPod (Cosmed 2007B, Concord, CA), which uses air displacement plethysmography. VAT,

subcutaneous adipose tissue (SAT), and hepatic fat for total liver volume (HF) were assessed via MRI at the UT-Austin Imaging Research Center on a research-dedicated Siemens Skyra 3 Tesla scanner utilizing a 3D 3-point DIXON technique. An average of 26 slices were taken from the abdominal area and there was no significant difference in the number of slices between groups. The liver was then manually segmented from the volume data utilizing MATLAB (Mathwork Inc, Natick, MA).[77] Fat volume was computed on a voxel-by-voxel basis and averaged over the segmented organ. At least two research assistants quantified the fat values for each subject. No significant differences in any of the outcome variables or MRI slices were seen between research assistants utilizing t-tests. Six subjects had unquantifiable MRI data (n=92). NAFLD was defined as subjects with >5.56% liver fat for total liver volume.[37–39]

Fasting Blood Draws

A fasting blood sample was obtained using a certified phlebotomist. Samples were spun to serum at the time of collection and frozen at -80 C in the freezers of Dr. Davis at the UT-Austin until the assays were performed by the Department of Medicine-Athero & Lipo in Baylor College of Medicine. Lipids were assayed using Vitros Colorimetric assays (Johnson and Johnson Clinical Diagnostics Rochester, NY) for cholesterol, triglycerides, low density lipids (LDL), and high density lipids (HDL). Free fatty acids were assayed using a colorimetric kit (NEFA-HR [2], Wako Diagnostics). Glucose was assayed on a Yellow Springs Instrument 2700 Analyzer (Yellow Springs, OH) using the glucose oxidase method. Insulin was assayed using a specific human insulin ELISA kit from (Linco, St. Charles, MO). Homeostatic model assessment (HOMA-IR) was calculated using the insulin resistance equation.[78,79] Adipokines such as

leptin, adiponectin, tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10), C-reactive protein (CRP) were measured using the Millipore Multiplex assay kit (EMD Millipore, Merck KGaA, Darmstadt, Germany). Six subjects had difficulties with the blood draw such as fainting or unable to locate a vein and un-readable blood measures in one subject (n=90).

Statistical Analysis

Data was examined for normality, and transformations were made if the data was found to be significantly different from normal. The following outcome variables were non-normally distributed and were either log, square root, or inversely transformed before the analysis: VAT, SAT, WC, HF, insulin, HOMA-IR, leptin, IL-6, IL-10, TNF-α, CRP, adiponectin, HDL, mean energy intake, percent dietary saturated fat, added sugar, and grams of total fiber, soluble fiber, and insoluble fiber. However, we present back-transformed[80] values in the tables and figures for ease of interpretation. Multiple analysis of covariance (MANCOVA) analyses were used to assess differences between dietary intake variables and adiposity and metabolic measures. Adiposity and metabolic variables were grouped based on significant correlations between variables as well as biological implications. The following variables were grouped and used as dependent variables: 1) adiposity measures included VAT, SAT, HF, total body fat, WC, and BMIz; 2) lipid measures included HDL, cholesterol, triglyceride, and LDL; 3) the glucose/insulin measures included glucose, insulin, and HOMA-IR; and 4) adipokines included leptin, IL-10, adiponectin, and IL-6, TNF-α, and CRP. Independent variables included the following macronutrients: fat, protein, carbohydrates, total sugar, added sugar, fiber, soluble fiber, and insoluble fiber. In all models the following *a priori*

covariates were included: total energy intake and sex.

If significant dietary variables were linked to hepatic fat in the MANCOVA models, then binary logistic regression was performed to assess relationship between diet and NAFLD. Chi-squared tests were performed on significant variables to test significance in mean consumption between groups. All analyses were performed by using SPSS version 20.0 (SPSS, Chicago, IL), and the significance was set at $p \leq 0.05$.

RESULTS

Participant Characteristics

Demographic data and adiposity measures are presented in **Table 3.1**. There were 98 subjects with complete dietary data, 92 with complete MRI and lipid data, and 90 with complete glucose/insulin and adipokine data. The sample was 50% female, 30.3% obese, 21.7% with NAFLD, and the average age was 18.8 years. The average total energy intake was approximately 2000 calories a day, dietary fat intake was 34.1% of total energy intake per day while average added sugar consumed was 70.7 grams per day.

Table 3.1: Demographics of participants

Subject & Diet Characteristics[a]	
Sex M/F	49/49
Age (y)	18.8 ±0.4
Height (cm)	167.6 ±9.7
Weight (kg)	67.0 ±13.7
Overweight/Obese Prevalence	30 (30.6)
NAFLD Prevalence	20 (20.4)
Total kilocalories (kcal)	1961.0 ±734.0
Dietary Protein (% daily kcal)	17.2 ±4.5
Dietary Fat (% daily kcal)	34.1 ±5.6
Dietary Saturated Fat (% daily kcal)	10.6 ±2.5
Carbohydrates (% daily kcal)	48.5 ±7.3
Added Sugar (g)	70.7 ±48.9
Total Fiber (g)	16.7 ±7.4
Adiposity and Metabolic Measures	
BMI (kg/m2) [b]	23.8 ±3.9
Visceral Adipose Tissue (ml)[b]	267.8 ±123.8
Subcutaneous Adipose Tissue (ml)[b]	999.7 ±654.8
Waist Circumference (cm) [b]	84.6 ±9.6
Hepatic Fat (%)[b]	5.9 ±5.2
Body Fat (%)[b]	26.4 ±9.8
BMI z score[b]	0.3 ±0.9
High Density Lipoprotein (mg/dl) [b]	54.8 ±11.7
Cholesterol (mg/dL) [b]	151.2 ±22.6
Triglyceride (mg/dL) [b]	81.5 ±35.4
Low Density Lipoprotein (mg/dL) [b]	79.9 ±18.9
Glucose (mg/dL)[c]	89.8 ±7.3
Insulin (μU/mL) [c]	8.8 ±6.0
HOMA-IR[c]	35.6 ±25.4
Leptin (ng/ml)[c]	18.0 ±17.0
IL-6 (pg/ml)[c]	1.6 ±1.4
IL-10 (pg/ml)[c]	0.5 ±0.4
TNF-α (pg/ml)[c]	6.1 ±2.5
CRP (mg/L)[c]	1.9 ±4.3
Adiponectin (mg/ml)[c]	12.7 ±6.0

Data presented as mean ± SD or n(%)
[a]n=98 [b]n=92 [c]n=90

Dietary Fat Parameters

Table 3.2 presents the relationship between adiposity and metabolic measures and total dietary fat, saturated fat, and protein intake. Total dietary fat intake was significantly associated with higher total body fat, SAT, fasting insulin, HOMA-IR, leptin, and CRP ($p<0.05$). Total dietary saturated fat was also associated with higher total body fat, SAT, HF, cholesterol, insulin, HOMA-IR, leptin, and CRP ($p<0.05$). Total protein intake was associated with higher CRP levels ($p<0.05$).

Table 3.2: MANCOVA analysis of the relationships between total dietary fat, saturated fat, and protein with adiposity and metabolic measures.

	Total Dietary Fat		**Total Dietary Saturated Fat**		**Protein**	
Adiposity	*β (95% CI)*	*p-value*	*β (95% CI)*	*p-value*	*β (95% CI)*	*p-value*
Visceral Adipose Tissue	1.009 (0.993, 1.024)	0.262	1.002 (0.998, 1.006)	0.280	0.018 (0.036, 0.001)	0.066
Subcutaneous Adipose Tissue	**1.028 (1.004, 1.051)**	**0.022***	**1.006 (1.001, 1.011)**	**0.030***	0.018 (0.046, 0.012)	0.238
Waist Circumference	0.000 (0.000, 0.000)	0.093	−10.00 (−100, −0.345)	0.060	0.000 (0.000, 0.000)	0.366
Hepatic Fat	−0.003 (−0.005, 0.000)	0.073	**0.114 (0.066, 0.417)**	**0.010***	0.003 (−0.001, 0.006)	0.100
Total Body Fat	**0.338 (0.024, 0.652)**	**0.035***	**0.093 (0.019, 0.167)**	**0.010***	0.035 (−0.359, 0.430)	0.859
BMIz	0.03 (−0.007, 0.067)	0.111	0.008 (−0.001, 0.017)	0.073	−0.009 (−0.055, 0.036)	0.686
Lipids						
HDL	1.003 (0.995, 1.010)	0.517	1.001 (0.999, 1.003)	0.276	0.004 (−0.005, 0.013)	0.402
Cholesterol	0.642 (−0.194, 1.477)	0.130	**0.289 (0.098, 0.479)**	**0.003***	0.180 (−0.786, 1.146)	0.712
Trigylceride	0.013 (−1.380, 1.405)	0.986	0.185 (−0.141, 0.512)	0.263	−0.974 (−2.553, 0.604)	0.223
LDL	0.316 (−0.416, 1.049)	0.393	0.163 (−0.008, 0.333)	0.061	0.192 (−0.648, 1.031)	0.651
Glucose/Insulin						
Glucose	0.225 (−0.048, 0.498)	0.105	0.008 (−0.057, 0.074)	0.804	−0.117(−0.434, 0.200)	0.466
Insulin	**1.033 (1.010, 1.057)**	**0.005***	**1.001 (1.004, 1.014)**	**0.001***	−0.010 (−0.037, 0.017)	0.473
HOMA-IR	**1.036 (1.012, 1.060)**	**0.003***	**1.009 (1.004, 1.015)**	**0.001***	−0.011 (−0.039, 0.017)	0.442
Adipokines						
Leptin	**0.008 (0.001, 0.021)**	**0.002***	**0.053 (0.010, 0.130)**	**0.001***	0.000 (−0.009, 0.001)	0.415
IL-6	.000 (0.000,0.001)	0.200	0.001 (0.000,0.004)	0.171	0.000 (0.000, 0.000)	0.659
IL-10	0.000 (0.000, 0.000)	0.255	0.000 (−0.001, 0.000)	0.479	0.000 (0.000, 0.000)	0.634
TNF-α	0.000 (0.000, 0.001)	0.089	0.000 (−0.001, 0.003)	0.684	0.000 (0.000, 0.000)	0.520
CRP	**1.060 (1.010, 1.111)**	**0.017***	**0.055 (0.018, 0.165)**	**0.003***	**−0.057 (−0.106, −0.003)**	**0.038***
Adiponectin	0.996 (0.981, 0.989)	0.597	0.008 (0.006, 0.012)	0.239	−0.004 (−0.022, 0.013)	0.620

*Indicates significance at $p<0.05$;

[a]Variables that were log transformed: BMI, VAT, SAT, HDL, Saturated Fat, CRP, Adiponectin;

[b]Variables that were inversely transformed: WC, HFF;

[c]Variables that were square-root transformed: Leptin, IL-6, IL-10, TNF-α;

[d]All analyses controlled for sex and total energy intake and reported in back-transformed value

Fiber Parameters

Table 3.3 presents the relationship between adiposity and metabolic measures with total dietary fiber, soluble fiber, and insoluble fiber. Increases in total dietary fiber and soluble fiber intake were inversely associated with hepatic fat, glucose, insulin, HOMA-IR, and leptin ($p<0.05$). Insoluble fiber intake was also inversely associated with hepatic fat, insulin, HOMA-IR, and leptin ($p<0.05$).

Table 3.3: MANCOVA analysis of the relationships between total dietary fiber, soluble fiber, and insoluble fiber with adiposity and metabolic measures.

	Total Dietary Fiber		**Total Dietary Soluble Fiber**		**Total Dietary Insoluble Fiber**	
Adiposity	*β (95% CI)*	*p-value*	*β (95% CI)*	*p-value*	*β (95% CI)*	*p-value*
Visceral Adipose Tissue	0.998 (0.996, 1.001)	0.164	0.998 (0.995, 1.000)	0.092	0.999 (0.996, 1.000)	0.222
Subcutaneous Adipose Tissue	0.997 (0.993, 0.999)	0.104	0.997 (0.993, 1.001)	0.095	0.997 (0.994, 0.999)	0.119
Waist Circumference	0.000 (0.000, 0.001)	0.407	0.000 (0.000, 0.001)	0.334	0.000 (0.000, 0.001)	0.417
Hepatic Fat	**−0.164 (−0.556, −0.095)**	**0.006***	**−0.156 (−0.588, −0.090)**	**0.009***	**−0.192 (−0.769, −0.110)**	**0.009***
Total Body Fat	−0.036 (−0.085, 0.014)	0.157	−0.035 (−0.089, 0.019)	0.197	−0.033 (−0.077, 0.011)	0.139
BMIz	−0.003 (−0.009, 0.004)	0.410	−0.003 (−0.010, 0.003)	0.340	−0.002 (−0.008, 0.003)	0.417
Lipids						
HDL	1.000 (0.999, 1.001)	0.706	1.000 (0.999, 1.001)	0.892	1.000 (0.999, 1.001)	0.706
Cholesterol	−0.025 (−0.159, −0.109)	0.713	−0.106 (−0.248, −0.037)	0.145	−0.002 (−0.117, −0.120)	0.980
Trigylceride	0.152 (−0.372, 0.068	0.173	−0.143 (−0.381, 0.095)	0.236	−0.130 (−0.325, 0.065)	0.189
LDL	0.002 (−0.116, 0.121)	0.968	−0.071 (−0.198, 0.055)	0.265	0.024 (−0.081, 0.128)	0.653
Glucose/Insulin						
Glucose	**−0.038 (−0.074, −0.002)**	**0.038***	**−0.048 (−0.087, −0.008)**	**0.018***	−0.030 (−0.063, −0.003)	0.072
Insulin	**−0.005 (−0.008, −0.002)**	**0.001***	**−0.005 (−0.008, −0.002)**	**0.002***	**−0.004 (−0.007, −0.002)**	**0.001***
HOMA-IR	**−0.005 (−0.008, −0.002)**	**0.001***	**−0.006 (−0.009, −0.001)**	**0.001***	**−0.005 (−0.008, −0.002)**	**0.001***
Adipokines						
Leptin	**−0.014 (−0.043, −0.001)**	**0.012***	**−0.021 (−0.058, −0.002)**	**0.004***	**−0.008 (−0.030, 0.000)**	**0.027***
IL-6	0.000 (−0.001, 0.000)	0.422	−0.001 (−0.003, 0.000)	0.094	0.000 (−0.001, 0.000)	0.711
IL-10	0.000 (−0.001, 0.000)	0.257	0.000 (−0.001, 0.000)	0.370	0.000 (0.000, 0.000)	0.226
TNF-α	0.000 (−0.002, 0.000)	0.418	0.000 (−0.002, 0.000)	0.416	0.000 (−0.001, 0.000)	0.451
CRP	0.000 (−0.007, 0.005)	0.888	0.005 (0.002, 0.011)	0.075	0.012 (0.006, 0.024)	0.623
Adiponectin	0.000 (−0.001, 0.000)	0.450	0.010 (0.007, 0.013)	0.786	0.009 (0.007, 0.011)	0.335

[*] Indicates significance at $p<0.05$;

[a] Variables that were log transformed: BMI, VAT, SAT, HDL, Total Fiber, Soluble Fiber, Insoluble Fiber, CRP, Adiponectin;

[b] Variables that were inversely transformed: WC, HFF;

[c] Variables that were square-root transformed: Leptin, IL-6, IL-10, TNF-α;

[d] All analyses controlled for sex and total energy intake and reported in back-transformed value

Carbohydrate Parameters

Table 3.4 presents the relationship between adiposity and metabolic measures with carbohydrates, total sugar, and added sugar intake. Total carbohydrate intake was inversely related to CRP levels ($p<0.01$). Total sugar intake was related to higher triglycerides ($p<0.05$) and added sugar intake was related to higher VAT ($p<0.05$).

Table 3.4: MANCOVA analysis of the relationships between carbohydrates, total sugar, and added sugar with adiposity and metabolic measures.

	Carbohydrates		**Total Sugar**		**Added Sugar**	
Adiposity	*β (95% CI)*	*p-value*	*β (95% CI)*	*p-value*	*β (95% CI)*	*p-value*
Visceral Adipose Tissue	0.002 (−0.010, 0.014)	0.717	0.002 (−0.002, 0.002)	0.667	**1.001 (1.000, 1.002)**	**0.046***
Subcutaneous Adipose Tissue	−0.009 (−0.027, 0.009)	0.346	−0.003 (−0.006, 0.000)	0.17	0.999 (0.997, 1.002)	0.553
Waist Circumference	0.000 (0.000, 0.000)	0.501	0.000 (0.000, 0.001)	0.341	0.000 (0.000, 0.000)	0.849
Hepatic Fat	0.000 (−0.002, 0.002)	0.802	−0.002 (−0.038, 0.035)	0.936	−0.014 (−0.042, 0.013)	0.302
Total Body Fat	−0.198 (−0.436, 0.039)	0.101	−0.035 (−0.075, 0.005)	0.085	−0.015 (−0.045, 0.014)	0.298
BMIz	0.012 (−0.04, 0.016)	0.396	0.001 (−0.006, 0.003)	0.473	0.000 (−0.004, −0.002)	0.692
Lipids						
HDL	−0.003 (−0.009, 0.003)	0.286	−0.018 (−0.077, 0.124)	0.715	0.999 (0.998, 1.000)	0.467
Cholesterol	−0.449 (−1.068, 0.169)	0.152	0.088 (−0.016, 0.193)	0.097	0.045(−0.033, 0.123)	0.255
Trigylceride	0.428 (−0.598, 1.454)	0.409	**0.175 (0.002, 0.348)**	**0.047***	0.102 (−0.028, 0.234)	0.124
LDL	−0.274 (−0.814, 0.267)	0.317	0.059 (−0.033, 0.152)	0.207	0.045 (−0.022, 0.115)	0.189
Glucose/Insulin						
Glucose	−0.060 (−0.265, 0.144)	0.558	−0.007 (−0.042, 0.027)	0.667	0.002(−0.023, 0.029)	0.830
Insulin	−0.013 (−0.030, 0.004)	0.137	−0.001 (−0.004, 0.001)	0.376	1.001 (0.998, 1.003)	0.340
HOMA-IR	−0.014 (−0.032, 0.004)	0.129	−0.002 (−0.005, 0.001)	0.358	1.001 (0.998, 1.003)	0.348
Adipokines						
Leptin	−0.001 (−0.006, 0.000)	0.1	0.002 (−0.016, 0.000)	0.149	0.000 (−0.006, 0.001)	0.436
IL-6	0.000 (0.000, 0.000)	0.237	0.000 (0.000, 0.000)	0.442	0.000 (0.000. 0.000)	0.422
IL-10	0.000 (0.000, 0.000)	0.635	0.000 (0.000, 0.000)	0.847	0.000 (0.000, 0.000)	0.850
TNF-α	0.000 (0.000, 0.000)	0.081	0.000 (0.000, 0.002)	0.101	0.000 (0.001, 0.000)	0.103
CRP	**0.054 (0.085, 0.021)**	**0.002***	0.006 (0.003, 0.008)	0.12	0.006 (0.004, 0.010)	0.071
Adiponectin	0.004 (−0.007, 0.016)	0.438	0.010 (0.008, 0.012)	0.877	0.010 (0.009, 0.012)	0.548

*Indicates significance at $p<0.05$;

[a] Variables that were log transformed: BMI, VAT, SAT, HDL, Total Sugar, Added Sugar, CRP, Adiponectin;

[b] Variables that were inversely transformed: WC, HFF;

[c] Variables that were square-root transformed: Leptin, IL-6, IL-10, TNF-α;

[d] All analyses controlled for sex and total energy intake and reported in back-transformed values

NAFLD

Students with NAFLD had a significantly higher intakes of saturated fat (11.89 ±0.71 % kcals versus 10.12 ±0.25 % of kcals , $p<0.001$) as seen in **Figure 2.2**. The odds of having NAFLD increase by 34% (95% CI: 1.08, 1.65, p=0.04) for every percent increase of dietary saturated fat.

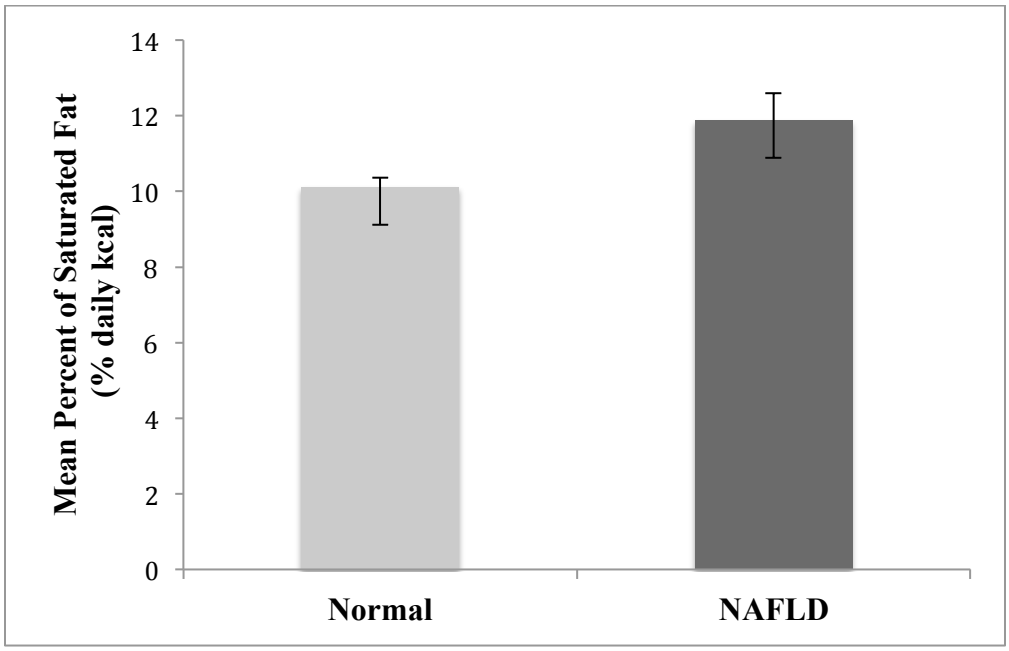

Figure 2.2: Dietary saturated fat intake in subjects with non-alcoholic fatty liver disease (NAFLD) was higher compared to normal subjects. Data presented as mean ±SD.

DISCUSSION

This is the first study to examine the relationship between dietary intake and adiposity and metabolic parameters in a Hispanic college population. These findings

support previous findings[49,81] that diets high in fiber are linked to lower adiposity and healthier metabolic outcomes while diets high in saturated fat and added sugar are related to higher adiposity and increased risk of metabolic diseases.[12,52,81,82]

Daily dietary fat percentages were high in this population, over 34%, which is near the upper limit of the Dietary Reference Intakes (DRI) recommendation for older children and adults of 25-35%. [83] Saturated fat percentages were above recommended ranges, with 10.6% of daily energy coming from saturated fat compared to the DRI recommendation for children and adults of <10%.[83] These dietary variables were related to increases in SAT, total body fat, insulin, HOMA-IR, leptin, and CRP and may be modulated by the same pathway. A prolonged high-fat diet has been linked to insulin-resistance, thereby requiring an increased production of insulin for glucose absorption.[84] In addition, excess dietary fat increases the size and count of adipocytes that release leptin to regulate body weight and weight gain.[85] Leptin has been shown to up-regulate CRP and indicates an immune response.[86]

Hepatic fat and cholesterol were only increased in the saturated fat variable and implies a specific pathway not associated with unsaturated fats. Furthermore, only dietary saturated fat was linked to increased odds of NAFLD. Previous cross-sectional studies and intervention studies in adults have found that saturated fat contributes to higher levels of adipokines, insulin resistance, and the development of NAFLD.[52,87,88] An intervention study showed that hepatic fat is directly modifiable by diet when a decrease of saturated fat in the diet contributed to a decrease in hepatic fat in overweight and obese women.[89] However, no other study has examined how diet relates to liver fat in a transitional and

high-risk population of college Hispanic freshmen.

The mechanisms behind dietary fat and hepatic fat storage remain unclear, but many theories exist. Mice studies collectively suggest that the liver is the first organ to store excessive amounts of fatty acids since a significant percentage is absorbed by the liver after a meal.[89] A high saturated fat diet may also be correlated with oxidative stress in the liver by impairing glutathione metabolism, and may cause an increased glucose-dependent insulinotropic polypeptide (GIP) response, which is associated with liver disease. [90] Therefore, excessive dietary saturated fat may first be transported to the liver and then oxidized causing an increase in inflammation and fat storage. In addition, CRP is produced by the liver so the increased levels of CRP, leptin, and hepatic fat support this mechanism.[91] However, CRP is produced in response to IL-6 levels, of which were not related to diet in this population.[91] The relationship between IL-6, CRP, and HF suggests that markers other than IL-6 may activate CRP.

On the contrary, students who ate more dietary protein had decreased levels of CRP. The breakdown of amino acids require energy and thereby may increase fat oxidation in the liver and decrease the inflammation markers such as CRP linked to increased levels of fat storage. In addition, derivatives of certain amino acids, such as taurine, may aid in the expression of regulating metabolic genes that decrease inflammation.[90] This is the first study to show that free-living protein intake is linked to lower CRP, and intervention and longitudinal studies should be conducted to further explore this relationship.

We also found that added sugar intake was linked to increased VAT in this population, which is consistent with our previous work in African American and Hispanic, overweight adolescents.[92] Added sugar in the Western diet is increasingly in the form of high fructose corn syrup, which has been shown to specifically up-regulate the expression of lipogenic genes that promote fat storage in VAT.[93,94]

Previously, we found an association between added sugar and insulin resistance in overweight Hispanic youth (ages 10-17 years).[15] However, in the current study no relation between added sugar and glucose/insulin action was found. Possible explanations are that the daily added sugar intake in the current population was fairly low, making up only 282 of daily calories, which is less than the mean added sugar intake in both boys (442 kcals/d) and girls (314 kcals/d) (12-19 years of age) in the US[95], and within the 5%-15% DRI recommended range.[83,95] Another explanation for the null findings is that this population included normal weight subjects, many who were insulin sensitive. Regardless, the rather low added sugar intake was linked to higher VAT.

Carbohydrate intake was linked to increased CRP, which was also seen in an observational study of 244 healthy women.[96] These data suggest that carbohydrates with a high glycemic load contribute to increases in pro-inflammatory processes. However, when isolating carbohydrates to total sugar, we found the only significant increase was in triglycerides, suggesting that carbohydrates may be contributing to excess energy intake that may lead to increased lipid storage that promotes overall inflammation. However, the mechanisms behind these findings need to be further explored.

Dietary fiber intake in this population was relatively low at 16.8 g/d, which is

below the DRIs ranges of 24-35 g/d, although in line with national averages for this age group.[83,97] Dietary fiber in this study was inversely linked to hepatic fat, fasting glucose and insulin, insulin resistance, and leptin. Possible explanations for these findings include phytochemicals within fibrous foods triggering anti-inflammatory pathways.[94] The gel-like properties of fiber may also moderate the glycemic response by slowing down gastric emptying, thereby improving insulin sensitivity and reducing the absorption of macronutrients and storage of hepatic fat.[95] The reduction of anti-inflammatory pathways and also the decrease in fat storage could collectively contribute to the decrease in leptin levels.[98] In addition, there is some evidence that fiber contributes to microbiotic expression of hormones, such as proglucagon derived peptide (GLP-2), which decreases gut permeability and hepatic inflammatory responses.[70] However, further research on the effects of fiber and the microbiome is needed.

Contrary to our previous findings and other studies [81,99–101] dietary fiber was not associated with VAT or inflammatory markers. However, this study differed from the other study populations in age, ethnicity, and weight status. One study was conducted with an older adult population, two studies used Norwegian young adults of varying BMI, and our previous study included both African American and Hispanic adolescents (14-18 years of age) who were all overweight or obese.

There are several limitations to mention. This study was cross-sectional and causality could not be determined. In addition, the sample size was relatively small, these students may be of higher socioeconomic status, and UT-Austin was ranked as a top 20 fittest campus in the US.[102] Our Hispanic young adults appear to be leaner and fitter and

may not be representative of the general Hispanic population as the prevalence of overweight/obesity was only 30% compared to 38% of Hispanic children (2-19 years of age) in the US.[11] However, the diets of this population were similar to the national diet averages.[97,103] As college enrollment in Hispanics continues to rise, it becomes increasingly important to understanding the health consequences of this population.

In conclusion, diets high in saturated fat and added sugar and low in dietary fiber, which is representative of the Western diet, were associated with increases in insulin resistance, inflammatory responses, and increased adipose tissue in specifically HF and VAT, and increased NAFLD in Hispanic college students. This time period is an important transitional period for influencing lifetime dietary habits and determinants of chronic disease. Reductions in dietary sugar and fat and increases in fiber may be potential targets for obesity intervention and prevention efforts in Hispanic populations.

Chapter 4: Altered composition of fecal microbiome associated with adiposity and metabolic parameters in Hispanic college students

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(In preparation for American Journal of Clinical Nutrition.)

ABSTRACT

Background: Hispanics are disproportionately affected by obesity and its accompanying morbidities such as type-2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and heart disease. All of these disease states have been linked to changes in the composition of the gut microbiome. No study has examined the relationship between the gut microbiome and cardiometabolic risk factors in an exclusively Hispanic young adult (18-19 y) population.

Objective: The purpose of this study is to examine the relationship between cardiometabolic risk markers and the gut microbiome in Hispanic college freshmen.

Design: BMI, waist circumference, body fat via BodPod, hepatic fat (HF), visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT) via magnetic resonance imaging, glucose, insulin, insulin resistance, and lipids via fasting blood draw, and stool samples via fecal wipe were collected in a cross-sectional study of 76 Hispanic college freshmen. Adiposity and metabolic variables were grouped according to tertiles and subjects were classified as having low, moderate, or high values. The microbiome was analyzed for diversity between groups. Significant variables were further analyzed for diversifying bacteria.

Results: Significant differences in microbial diversity were found between tertiles of total body fat, low-density lipoprotein (LDL), and insulin as determined from unweighted

Uni-Frac analysis. Those subjects with high body fat and insulin compared to those with low body fat and insulin had significantly less overall diversity. Those subjects with low values of LDL compared to those with high LDL levels had significantly less overall diversity.

Conclusion: Few human studies have assessed the relationship of adiposity and cardiometabolic risk factors with microbiome diversity, and this is the first study to examine this relationship in an exclusive healthy Hispanic college population. This study suggests that by increasing biodiversity of the microbiome we may thereby decrease inflammatory diseases.

INTRODUCTION

The gut microbiome is a diverse and dynamic community of microbes within the human gastrointestinal (GI) tract whose structure and composition plays a role in metabolism and energy homeostasis.[18] What constitutes a "healthy" gut microbiome remains unknown, but current research suggests diversity of microbial populations is essential. Decreases in microbial diversity have been demonstrated in disease states, including obesity, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease.[18–20,61,104,105]

Hispanics are disproportionately affected by obesity, T2D, and NAFLD.[11] They are also the largest and fastest growing ethnic minority in the US and in recent history have surpassed Non-Hispanic Whites and Blacks in college enrollment.[1,2] In 2016,

Hispanic students represented 23% of freshman enrollment at the University of Texas at Austin (UT-Austin), having the largest increase among all minority groups.[106]

Given this population is disproportionately affected by obesity and metabolic disease, and that the college years are a critical transition period in which lifestyle habits are established, understanding how adiposity and metabolic markers are linked to the gut microbiome in this population is warranted. Thus, the **overall goal** of this study is to examine the relationship between adiposity and metabolic markers and the gut microbiome in Hispanic college freshmen. Subjects with healthy ranges of adiposity and metabolic markers are hypothesized to have a greater diversity than subjects outside of healthy ranges.

SUBJECTS AND METHODS

Participants

Figure 4.1 provides a detailed flow of study participants. The original purpose of this study was to examine the relationship between eating frequency and adiposity and metabolic markers. Hispanic college freshmen subjects were recruited via announcement in classes, word of mouth, electronic posted notices, and tabling at dorms around the UT-Austin campus. Subjects completed a screener to determine eligibility. Inclusion criteria included: (i) self-report that all four of their grandparents were of Hispanic origin (ii) 18-19 years of age, and (iii) in their first year of college. Exclusion criteria included (i) current pregnancy, (ii) taking any medication known to affect body composition or any psychoactive medication, (iii) diagnosis with a disease(s) or syndrome known to affect

body composition or fat distribution, (iv) if they had a learning impairment(s) that would complicate survey administration, (v) braces, a pacemaker, or any other contraindications to magnetic resonance imaging scanning, or (vi) participation in a weight loss, dietary, or physical intervention in the previous six months. Of the 791 eligible Hispanic students, diet recalls were conducted in 100 subjects for the original purpose of this study, and one subject was excluded due to extremely low carbohydrate intake of less than 5% of total daily intake. Seventy-eight of the remaining subjects contributed a fecal sample, but 76 were analyzed due to unreadable labels on two of the samples. Adiposity measures were taken via MRI and blood glucose and lipids were taken via fasting blood draw. Three subjects had an unquantifiable MRI, five had blood draw difficulties, and one had an unreadable glucose assay. Therefore, 73 subjects had complete adiposity measures, 71 had complete lipid measures, and 70 had complete glucose measures.

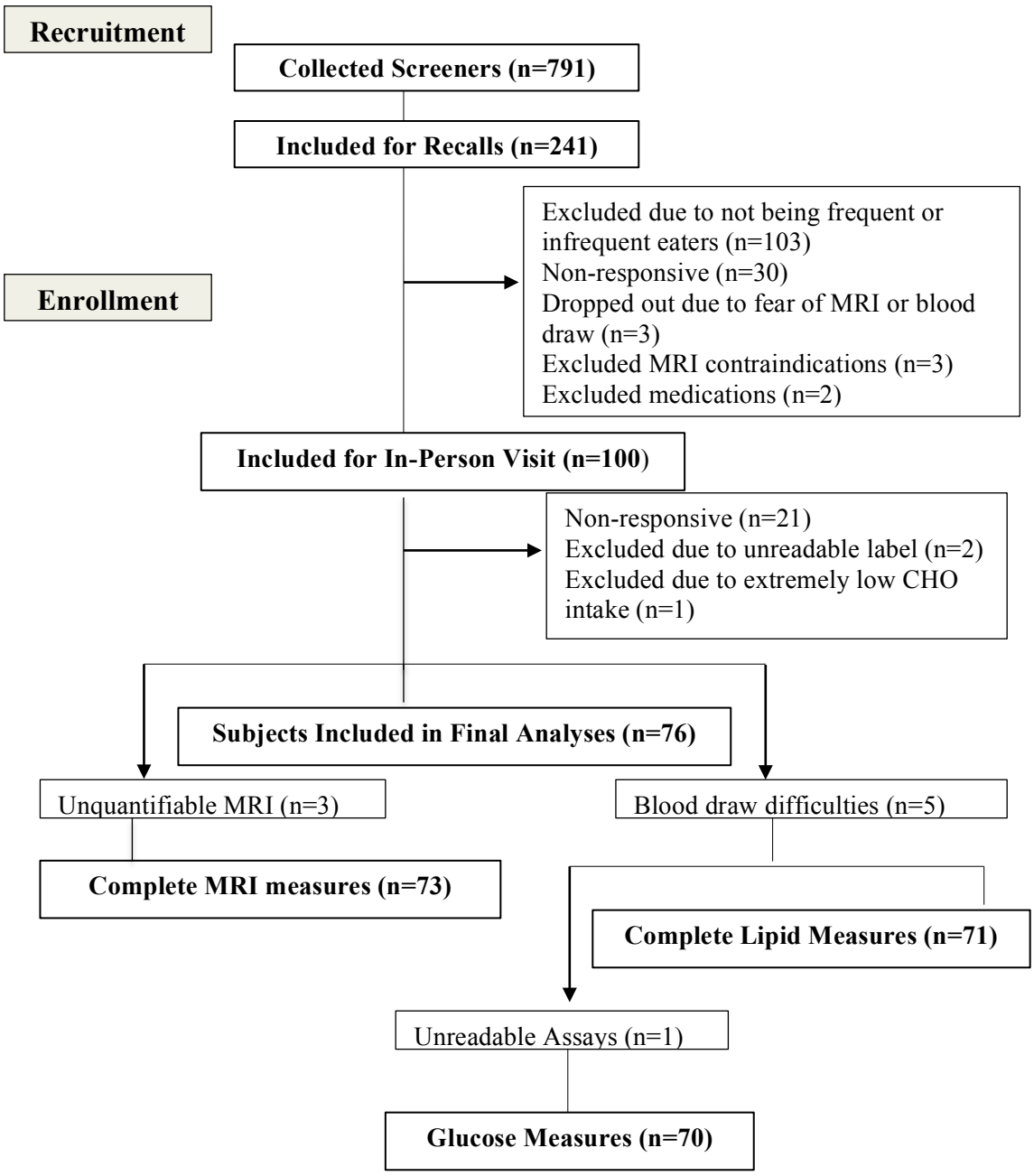

Figure 4.1: Recruitment and enrollment of study participants

Anthropometrics and Adiposity Measures

Height and weight were measured to the nearest 0.1 kg and 0.1 cm using a beam

medical scale and a wall-mounted stadiometer, respectively, and the average of two measurements was used for the analysis. BMI was calculated utilizing adult cut offs and body mass index (BMI) percentiles and z-scores (BMI-z) were determined by using EPII 2000 software (version 1.1; Centers for Disease Control and Prevention, Atlanta, GA). Subjects were categorized as overweight if they had a BMI of 25.0 to < 30.0 and obese if they had a BMI >30.0. Waist circumferences (WC) were measured and recorded to the nearest 0.1 cm. Body fat and soft lean tissue were measured using the BodPod (Cosmed 2007B, Concord, CA), which uses air displacement plethysmography. VAT, subcutaneous adipose tissue (SAT), and hepatic fat for total liver volume (HF) were assessed via MRI at the UT-Austin Imaging Research Center on a research-dedicated Siemens Skyra 3 Tesla scanner utilizing a 3D 3-point DIXON technique. An average of 26 slices were taken from the abdominal area and there was no significant difference in the number of slices between groups. The liver was then manually segmented from the volume data utilizing MATLAB (Mathwork Inc, Natick, MA).[77] Fat volume was computed on a voxel-by-voxel basis and averaged over the segmented organ. At least two research assistants quantified the fat values for each subject. No significant differences in any of the outcome variables or MRI slices were seen between research assistants utilizing t-tests. NAFLD was defined as subjects with >5.56% liver fat for total liver volume.[37–39]

Fasting Blood Draws

A fasting blood sample was obtained using a certified phlebotomist. Samples were spun to serum at the time of collection and frozen at -80 C in the freezers of Dr. Davis at the UT-Austin until the assays were performed by the Department of Medicine-Athero & Lipo in Baylor College of Medicine. Lipids were assayed using Vitros Colorimetric assays (Johnson and Johnson Clinical Diagnostics Rochester, NY) for cholesterol, triglycerides, low-density lipids (LDL), and high density lipids (HDL). Glucose was assayed on a Yellow Springs Instrument 2700 Analyzer (Yellow Springs, OH) using the glucose oxidase method. Insulin was assayed using a specific human insulin ELISA kit from (Linco, St. Charles, MO). Homeostatic model assessment (HOMA-IR) was calculated using the insulin resistance equation.[78,79]

Stool Samples

Participants were given a kit for stool sample collection with instructions and a brief questionnaire regarding past gastrointestinal illness, antibiotic use, and supplement use within one week of their in-person visit. Participants were instructed to collect the first stool of the day with a pre-moistened sample wipe, which was stored in a labeled biosafety bag and picked up by research staff within 24-hours of collection. Seventy-nine subjects returned a stool sample. Two of those subjects were excluded due to unreadable identifiers on the stool sample (n=77). Fecal DNA was stored in a -80 freezer and extracted by trained research staff using the Fecal DNA Isolation Kit (Zymo Research,

cat. no. D6010. Coded specimen was then shipped to the Microbiome Resource Laboratory (Birmingham, Alabama) to undergo 16S ribosomal RNA gene sequencing.[66]

The DNA was amplified via polymerase chain reaction (PCR) using degenerate primers flanking the V4 region of the rRNA gene to generate a 250 base pair amplicon. The individual samples were electrophoresed on agarose gel and visualed by UV illumination. The PCR product was excised and purified using a commercial (QIA) gel extraction kit (Qiagen, cat. No. 28704). The purified PCR products were then quantitated using Pico Green dsDNA reagent then sequenced using the NextGen sequencing Illumina Miseq platform from both the 5' prime and the 3' end.[66]

Microbial Composition Analysis

Fecal microbiome composition was analyzed in the microbiome analysis package Quantitative Insights Into Microbial Ecology (QIIME) v1.9.0 and simplified with QWRAP, an online statistical software tool to test differences in the microbe composition within groupings.[66] Standard methods were performed for quality control, generating abundance, descriptive statistics of sample bacteria, significant differences between groups, and the specific bacteria between the groups contributing to those differences.[64] Within QWRAP, a quality control check was done using FASTQC v0.11.2 and FASTX v0.0.13 in which all of the raw data are trimmed to reads with over at least 80% base-pairs retained.[66] Counts per sample ranged from 12,700 to 187,656. Clusters of reads with sequence similarity above a 97% cutoff were binned into Operational Taxonomic Units (OTUs), which were counted and used to measure relative abundance.[64,66] Rarefaction

curves were generated to ensure sufficient depth and measure alpha diversity.[64,66] A PERMANOVA compared the alpha diversities between tertiles with Monte Carlo permutations.[66] A p-value of < 0.05 was used to determine significance. Unweighted UniFrac, weighted UniFrac, and Bray-Curtis analysis was used to estimate beta diversity, which calculates distance as a measure of similarity of microbial communities between samples or groups of samples.[66] Kruskal-Wallis tests were used to identify differential abundant bacterial phylotypes. A p-value of <0.05 with a false discovery rate (FDR) of <0.2, as was used in the Human Microbiome Project (HMP),[19] determined significance in order to adjust for multiple hypothesis tests.[107] Microbiome composition was then examined for differences between tertiles of adiposity and metabolic measures.

Adiposity and Metabolic Groupings

Adiposity and metabolic measures were split into tertiles using SPSS version 20.0 (SPSS, Chicago, IL) and displayed in **Table 4.1**. Adiposity and metabolic measures were also grouped according to any available recommendations of healthy values for this age group and listed in **Table 4.2**.[108–111] The classifications were performed separately by two different researchers and compared for reliability.

Table 4.1: Adiposity and cardiometabolic tertile cut-offs

Adiposity & Cardiometabolic Variables	Low Range	Moderate Range	High Range
Adiposity			
VAT (ml)	< 186.7	186.7–257.7	> 257.3
SAT (ml)	< 640.9	640.9–992.1	> 992.1
WC (cm)	< 80.3	80.3–85.2	> 85.2
HF (%)	< 4.1	4.1–5.0	> 5.0
Body Fat (%)	< 22.6	22.6–30.5	> 30.5
Lipids			
HDL (mg/dL)	< 51.2	51.2–59.3	> 59.3
Cholesterol (mg/dL)	< 138.9	138.9–165.8	> 165.8
Triglyceride (mg/dL)	< 60.0	60.0–89.8	> 89.8
LDL (mg/dL)	< 74.0	74.0–89.9	> 89.9
Glucose/Insulin			
Glucose (mg/dL)	< 87.0	87.0–92.0	> 92.0
Insulin (mU/dL)	< 6.2	6.2–10.0	> 10.0
HOMA-IR	< 1.39	1.3–2.1	> 2.1

Table 4.2: Recommendations for adiposity and cardiometabolic variables

Adiposity & Cardiometabolic Variables	Healthy Range	n (%)
Adiposity Anthropometrics (n=76)[a]		
WC (cm)	M: < 88.7 / F: < 83.1	46 (61)
BMI	Normal weight	57 (75)
Body Fat (%)[b]	M: < 29.5 / F: < 41.0	66 (80)
Adiposity MRI (n=73)		
NAFLD	No disease	57 (78)
Lipids (n=71)		
HDL (mg/dL)	> 40 mg/dL	67 (96)
Cholesterol (mg/dL)	< 200 mg/dL	69 (97)
Triglyceride (mg/dL)	< 150 mg/dL	68 (96)
LDL (mg/dL)	< 130 mg/dL	71 (100)
Glucose/Insulin (n=70)		
Glucose (mg/dL)	< 100 mg/dL	65 (93)
Insulin (mU/mL)	< 25 mU/mL	68 (97)
HOMA-IR: American	< 2.6	58 (83)
HOMA-IR: Mexican-American	< 3.8	68 (94)

[a] No recommendations for VAT, SAT [b] Recommendations specific to Mexican-Americans aged 16-19

RESULTS

Demographics and cardiometabolic measures are displayed in **Table 4.3**. The average age of participants was 18 years old, 45% were male, 25% were overweight or obese, and 22% had NAFLD. Averages of all the other adiposity and cardiometabolic variables were within the acceptable ranges for this age group.[108,111–113]

Table 4.3: Recommendations for adiposity and cardiometabolic variables. Data presented in mean ±SD or n (%)

Subject Characteristics (n=76)	
Sex M	34 (44.5%)
Age (y)	18.7 ±0.4
Overweight or Obese Prevalence	19 (25%)
Body Fat (%)	26.6 ±10.0
Waist Circumference (cm)	84.4 ±9.7
Adiposity (n=73)	
Visceral Adipose Tissue (ml)	253.4 ±128.2
Subcutaneous Adipose Tissue (ml)	973.37 ±637.6
Hepatic Fat Fraction (%)	5.9 ±5.5
Prevalence of NAFLD (%)	16 (22%)
Lipids (n=71)	
HDL (mg/dL)	55.0 ±11.3
Cholesterol (mg/dL)	153.0 ±22.4
Triglyceride (mg/dL)	80.7 ±33.7
LDL (mg/dL)	81.7 ±19.1
Glucose/Insulin (n=70)	
Glucose (mg/dL)	89.4 ±7.8
Insulin (mU/mL)	8.6 ±4.9
HOMA-IR	1.1 ±1.3

Body fat, LDL, and insulin had significant beta-diversity between groups (unweighted 0.02, 0.02, and 0.04, respectively). The mean values of significant tertiles were as follows: body fat– low: 15.7 ±5.0%, moderate: 26.2 ±2.4%, and high: 38.0 ±4.8%; LDL– low: 60.7 ±9.2 mg/dL, moderate: 81.7 ±4.5 mg/dL, and high: 102.7 ±9.7 mg/dL; insulin– low: 4.3 ±1.3 mU/dL, moderate: 7.9 ±1.2 mU/dL, and high: 13.6 ±5.4 mU/dL. Tertiles of VAT, SAT, WC, HF, HDL, cholesterol, triglyceride, glucose, and HOMA-IR did not achieve significance. Bray-Curtis and weighted Unifrac p-values did

not achieve significance.

Figure 4.2 displays the significant difference in alpha-diversity in the gut microbiome between tertiles of percent body fat, insulin, and LDL. Subjects with high body fat percent compared to those with low and moderate body fat percentages had lower alpha-diversity levels (4.8 ±0.8 vs. 5.1 ±0.7 and 5.2 ±0.5; p=0.02). Subjects with high and moderate insulin levels compared to those with low insulin values had lower alpha diversity levels (4.9 ±0.9 and 4.9 ±0.6 vs. 5.3 ±0.6; p=0.02). Subjects with high LDL levels compared to subjects with low and moderate LDL had increased diversity (5.3 ±0.6 vs. 4.9 ±0.5 and 5.0 ±0.9; p=0.04).

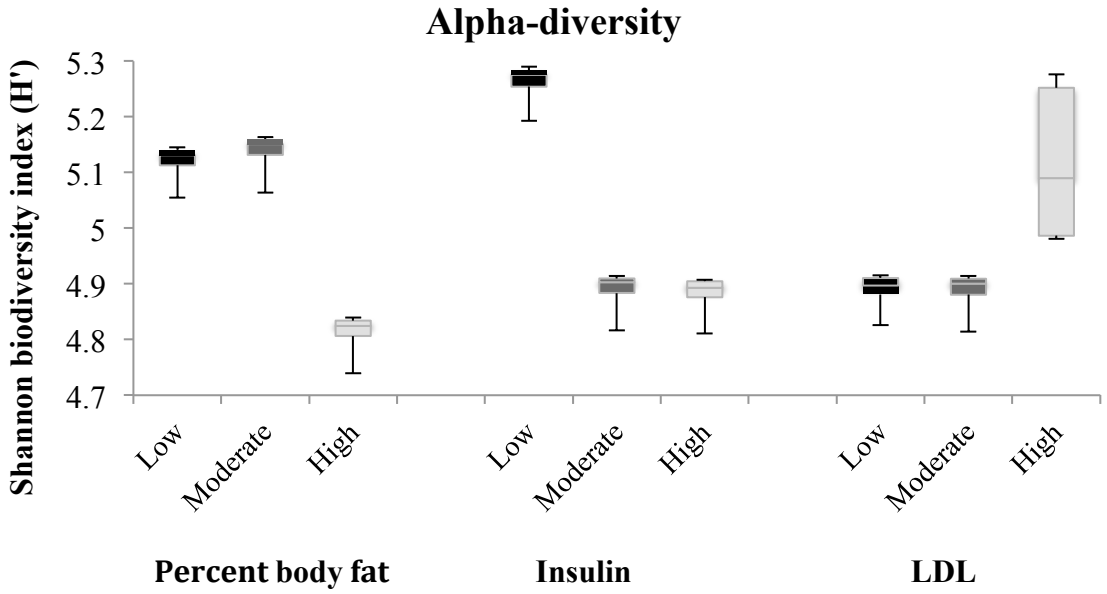

Figure 4.2: Microbiome alpha-diversity between subjects. Subjects with low percent body fat and low levels of insulin had a more diverse microbiome compared to subjects with high percent body fat and high insulin levels (p=0.02 and p=0.04, respectively). Subjects with high levels of LDL had a more diverse microbiome compared to subjects with low LDL levels (p=0.02).

Participants classified as either healthy or unhealthy using the recommendation parameters and the majority of subjects achieved the healthy recommendations. None of the variables achieved significance including NAFLD and BMI.

Table 4.4 displays the bacteria by taxonomic classification that are significantly different between tertiles of percent body fat. Bacteria at the species level did not achieve significance. Participants with high percent body fat compared to participants with low and moderate body fat percentages had significantly less relative abundance of *Methanobrevibacter*, *Akkermansia,* and *Clostridiales* and significantly more relative abundance of *Fusobacteriales* and *Bacteroidia*.

Table 4.4: Differences in bacteria between tertiles of body fat.

Taxonomic Classification	Bacteria	Mean relative abundance (%)			*p* value	*FDR*
		Low	Moderate	High		
Phylum	*Euryarchaeota*	0.00036	0.00038	0.00005	0.00	0.02
Phylum	*Verrucomicrobia*	0.01035	0.00268	0.00057	0.00	0.02
Phylum	*Fusobacteria*	0.00649	0.00131	0.00420	0.02	0.09
Phylum	*Bacteroidetes*	0.00090	0.00112	0.00084	0.04	0.09
Class	*Methanobacteria*	0.00036	0.00038	0.00005	0.00	0.03
Class	*Verrumicrobiae*	0.01010	0.00247	0.00056	0.00	0.03
Class	*Fusobacteriia*	0.00064	0.00131	0.00420	0.04	0.16
Class	*Bacteroidia*	0.30292	0.31076	0.38727	0.04	0.16
Class	*Clostridia*	0.58170	0.57095	0.47327	0.02	0.15
Order	*Methanobacteriales*	0.00036	0.00038	0.00005	0.00	0.04
Order	*Verrucomicrobiales*	0.01011	0.00247	0.00056	0.00	0.04
Order	*Fusobacteriales*	0.00064	0.00131	0.00420	0.02	0.19
Order	*Clostridiales*	0.58170	0.57095	0.47327	0.02	0.19
Family	*Methanobateriaceae*	0.00036	0.00038	0.00005	0.00	0.09
Family	*Verrucomicrobiaceae*	0.01010	0.00247	0.00056	0.00	0.09
Genus	*Methanobrevibacter*	0.00036	0.00038	0.00005	0.00	0.19
Genus	*Akkermansia*	0.01010	0.00247	0.00005	0.00	0.19

Methanobrevibacter belongs to family *Methanobateriaceae*, order *Methanobacteriales*, class *Methanobacteria*, and phylum *Euryarcheota*; *Akkermansia* belongs to family *Verrucomicrobiaceae*, order *Verrucomicrobiales*, class *Verrucomicrobiae,* and phylum *Verrucomicrobia*; and *Clostridiales* belongs to class *Clostridia*; *Fusobacteriales* belongs to class *Fusobacteriia* and phylum *Fusobacteria*; and *Bacteroidia* belongs to the phylum *Bacteroidetes;* all of which achieved significance.

Participants with high and low levels of insulin compared to participants with moderate levels of insulin had significantly less relative abundance of phylum *Proteobacteria* as displayed in **Table 4.5**.

Table 4.5: Differences in bacteria between tertiles of insulin.

Taxonomic Classification	Bacteria	Mean relative abundance (%)			*p* value	*FDR*
		Low	Moderate	High		
Phylum	*Proteobacteria*	0.04171	0.21222	0.03874	0.02	0.15

Table 4.6 lists the bacteria by taxonomic classification that are significantly different between tertiles of LDL. Participants with high levels of LDL compared to participants with low and moderate levels of LDL had significantly higher relative abundance of *anthropi, europaeus, Pyramidobacter*, *Mobilincus, Campylobacter*, *Facklamia, Gallicola*, *WAL_1855D*, *1-68*, *Mogibacterium*, and *Peptococcus*.

Table 4.6: Differences in bacteria between tertiles of LDL.

Taxonomic Classification	Bacteria	Mean relative abundance (%)			*p* value	*FDR*
		Low	Moderate	High		
Phylum	*Synergistetes*	0.00014	0.00000	0.00126	0.00	0.02
Class	*Synergistia*	0.00014	0.00000	0.00126	0.00	0.04
Class	*Epsilonproteobacteria*	0.00243	0.00184	0.01016	0.01	0.19
Order	*Synergistales*	0.00014	0.00000	0.00126	0.00	0.03
Family	*Dethiosulfovibrionaceae*	0.00014	0.00000	0.00126	0.00	0.07
Family	*Aerococcaceae*	0.00014	0.00118	0.00137	0.00	0.07
Genus	*Jonquetella*	0.00000	0.00000	0.00082	0.00	0.18
Genus	*Pyramidobacter*	0.00014	0.00000	0.00043	0.01	0.18
Genus	*Campylobacter*	0.00243	0.00184	0.01015	0.01	0.18
Genus	*Facklamia*	0.00013	0.00114	0.00111	0.01	0.18
Genus	*Mobiluncus*	0.00049	0.00074	0.00457	0.00	0.08
Genus	*Gallicola*	0.00051	0.00021	0.00141	0.01	0.18
Genus	*WAL_1855D*	0.01474	0.01084	0.03263	0.01	0.18
Genus	*1-68*	0.00500	0.00403	0.00786	0.01	0.18
Genus	*Moryella*	0.00009	0.00000	0.00048	0.01	0.18
Genus	*Mogibacterium*	0.00112	0.00056	0.00140	0.02	0.18
Genus	*Peptococcus*	0.00060	0.00035	0.00161	0.02	0.19
Species	*anthropi*	0.00000	0.00000	0.00082	0.00	0.19
Species	*europaeus*	0.00003	0.00002	0.00321	0.00	0.14

Species *anthropic* belongs to genus *Jonquetella*, family *Dethiosulfovibrionaceae*, order *Synergistales,* class *Synergistia,* and phylum *Synergistetes*. Genus *Pyramidobacter* belongs to the same taxonomic classifications. Genus *Campylobacter* belongs to class *Epsilonproteobacteria*. Genus *Facklamia* belongs to family *Aerococcaeceae*. All of the aforementioned achieved significance.

DISCUSSION

This is the first study to examine the relationship between cardiometabolic measures and the microbiome in an exclusively Hispanic college population.

Approximately one quarter of this population was overweight or obese and had NAFLD. Percent body fat, insulin, and LDL were the main variables linked to an altered microbiome. Subjects with high percent body fat compared to those with low and moderate percent body fat had lower microbial composition diversity, confirming several studies in which obesity was associated with a significant decreased level of diversity.[24,30,75,114] Subjects with high insulin levels compared to subjects with moderate and low insulin levels also had lower microbial composition diversity. This was expected since dysbiosis and decreases in microbial biodiversity have been associated with metabolic disease states, including diabetes.[22,31,70]

Contrary to our hypothesis, participants with low and moderate LDL levels compared to subjects who had high values had lower diversity. However, upon further analysis, the range of the LDL was large and the mean of the high LDL group was near optimal range with 100% of subjects meeting the recommendations for LDL. Some research suggests that low levels of LDL are correlated with adverse health effects.[115–117] Since LDL is a carrier protein for cholesterol, which is a critical component of cell membranes and sex hormones, subjects closer to the optimal range of LDL would have an increased microbial diversity. A more unhealthy population is needed to study the impact beyond low and optimal levels of LDL.

An increase of percent body fat was associated with lower relative abundance of *Methanobrevibacter, Akkermansia*, and *Clostridiales*. These bacteria have been classified as components of a healthy gut. In recent human studies, *Methanobrevibacter* has been associated with leanness, and is found to be prevalent in the healthy human colon

compromising up to 10% of the microbiome.[118] *Akkermansia* is a mucin-degrading bacteria that represents 3-5% of the microbial community in healthy subjects and is inversely correlated with body weight.[114,119,120] In addition, probiotic administration of *Akkermansia* was found to restore abundance and improve gut barrier and metabolic parameters in diet-induced obese mice.[119] *Clostridiales* has important roles in the metabolism of dietary fiber and was identified as the most active microbial component in healthy adult intestinal environments.[121–123] The current study confirms these findings and suggests that certain gut bacteria act as protective barriers against obesity.

Moderate values of insulin were associated with a decreased relative abundance of *Proteobacteria* compared to both high and low value groups. However, *Proteobacteria* is a phyla that encompasses bacteria of extreme metabolic diversity.[124] Therefore, no conclusions about *Proteobacteria* and insulin can be made without further specificity. This inconclusive data is most likely due to the participants meeting the recommended range of insulin, and therefore may all have similar microbes that aid in insulin management.

Low values of LDL were associated with decreased relative abundance of *Jonquetella anthropi, Actinomyces europaeus, Pyramidobacter, Peptococcus, Moryella, Mobilincus,Campylobacter, Facklamia, Mogibacterium, Gallicola, WAL_1855D,* and *I-68*. Some of these bacteria have designated functions, while the functions of others have not yet been found or are not clear. *Jonquetella anthropic, Actinomyces europaeus*, and *Pyramidobacter* aid in producing short chain fatty acids which are metabolically beneficial by decreasing inflammation.[61,125–128] *Peptococcus* are involved in circulation of

steroid molecules from the liver, which is the function of LDL and attests to a possible mechanism. *Moryella* ferment carbohydrates to produce indoles, which have diverse biological roles such as intercellular signaling.[129,130] *Mobilincus, Campylobacteria, Facklamia, Mogibacterium, Gallicola, WAL_1855D,* and *I-68* have unclear functions. It is important to note, that some of these bacteria have been associated with infections such as diarrhea or bacterial vaginosis, but their role or proof of pathogen has not been proven and is speculative.[131–134] These results suggest that having too low values of LDL may negatively affect the microbiome or vice versa. Having ranges near the optimal cut off is correlated with having non-inflammatory metabolic products. However, a wider range of LDL values are needed to draw conclusions between healthy and unhealthy subjects. In addition, the specific functions of bacteria in humans remain elusive and further examination is needed.

There were no significant differences in the gut microbiome between tertiles of VAT, SAT, WC, and HF. Participants who were overweight or obese compared to normal weight participants did not have differences in the gut microbiome. Of note, this population was healthier than most. Only 25% were classified as overweight or obese, which is significantly less than the national average for Hispanics in this age range of 38.9%.[2] In addition, over 96% of subjects met the recommend ranges for lipids and over 83% for insulin resistance. None of the subjects had metabolic syndrome or diabetes. Percent body fat did achieve significance and is a more comprehensive and direct measure for body fat.

There were no differences in gut microbiome between participants with NAFLD

and those without. NAFLD has been associated with an altered gut microbiome.[67] Although, like the current study, several human studies confirm there is no significant difference in overall diversity of microbial composition diversity between participants with NAFLD and their controls.[42,43] When further examined, the only significant bacteria in this population was the order *Verrucomicrobiales* and subjects with NAFLD had lower amounts. This bacterium is part of the phylum *Verrucomicrobia,* which only has two species detected in the human gastrointentstinal tract. A decreased abundance in those species are linked to compromised health.[134] However, this bacteria was not significant in other studies and participants with NAFLD have shown different abundances of bacteria for different ages and ethnic groups.[40,43]

There were no significant differences in cholesterol, triglyceride, HDL, glucose, and insulin resistance. An animal study showed the gut microbiome was a moderating link between dietary choline and the progression of atherosclerosis and increased cholesterol[105]. So although HDL was approaching significance, these null results are most likely due to this population being within recommended ranges for these measures.

The limitations of this study include its cross-sectional design and small sample size. This population was homogenous in ethnicity, location, and age, eliminating possible confounders which could be a strength but also a possible weakness. In addition, this population being metabolically healthy did not provide a contrast to unhealthy groups. A larger sample size, more diversity in metabolic values, and deeper amplification are needed to further explore significant differences in specifying bacteria available in smaller amounts. Also, the specific functions of bacteria need further

exploration.

In summary, few studies have implicated human cardiometabolic measures on the decreased diversity of the microbiome. Metabolic diseases disproportionally affect Hispanics and no studies have examined adiposity and metabolic measures on the microbiome in an exclusively Hispanic college population. [11] These findings suggest that participants with lower percentages of total body fat, lower values of insulin, and optimal values of LDL contain a higher relative abundance of a multitude of beneficial bacteria than those with more total body fat and insulin and low values of LDL. These bacteria suggest a possible mechanism for protection against cardiovascular and metabolic disease. More longitudinal and intervention studies are warranted to understand the role that diet plays on the gut microbiome and subsequent disease risk.

Chapter 5: Saturated fat intake correlates with altered composition of fecal microbiome in Hispanic college students

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(In preparation for American Journal of Clinical Nutrition.)

ABSTRACT

Background: The college years is a critical transition period in which lifetime eating habits are established and Hispanics are disproportionately affected by obesity and related metabolic disease. Significant and meaningful changes in the gut microbiota have been associated with dietary alterations, primarily consumption of dietary fiber, fat, sugar, and being breastfed during infancy, but the mechanism and magnitude of influence is unknown. In addition, the microbiome of an exclusive Hispanic population as well as college-aged students have not been studied. Therefore, understanding how diet impacts the composition of the fecal microbe community in Hispanic college students is warranted.

Objective: The purpose of this study is to examine the relationship between diet and the gut microbiome in Hispanic college freshmen.

Design: Dietary intake via multiple 24-hour dietary recalls and stool samples were collected from 76 Hispanic college freshmen (18-19 y). Dietary variables were grouped according to current recommendations and subjects were classified as either met recommendations or exceeded recommendations.

Results: Sixty-two percent of subjects exceeded the recommendations for saturated fat. Significant differences in microbial diversity were found between those who met recommendations and those who exceeded recommendations for saturated fat as

determined from unweighted Uni-Frac analysis. Those who exceeded saturated fat recommendations compared to those who met them had significantly less relative abundance of *Methanobacteriales, Victivallales, Bacillales, and Campylobacteria*.

Conclusion: Few human studies have collected extensive dietary data and microbiome diversity, and this is the first study to examine the relationship between dietary intake and the microbiome in an exclusively Hispanic college population. Our findings support previous findings in animal models that diets high in saturated fat correlated with decreased diversity in the microbial composition reflected by increased abundance of *Firmicutes*. This study suggests that a decrease in saturated fat intake is recommended in order to increase biodiversity and thereby decrease inflammatory diseases.

INTRODUCTION

College is known to be a transitional period of time when young adults in the United States (US) consume more junk food and alcohol, and less dietary fiber, fruits and vegetables.[4–6] Several studies have shown that 70% of college freshmen gain an average of 3.5 to 7.7 pounds in the first year of college[4,7–9] with no difference in dietary intake and physical activity from their freshman year to their senior year.[4,10] Therefore, the transition to college has been identified as a critical period contributing to the rise in obesity rates as the behavioral choices college students make likely affect their risk of chronic disease later in life.

Hispanics are the largest and fastest growing ethnic minority in the US and in recent history have surpassed Non-Hispanic Whites and Blacks in college enrollment.[1,2]

In 2016, Hispanic students represented 23% of freshman enrollment at the University of Texas at Austin (UT-Austin), having the largest increase among all minority groups.[106] Hispanics are also disproportionately affected by obesity, type-2 diabetes (T2D), and non-alcoholic fatty liver disease.[11] Diets high in added sugar and low in dietary fiber as well as decreased eating frequency and skipping breakfast have been positively linked to obesity levels, visceral adipose tissue, insulin resistance, and circulating lipids in Hispanic youth and young adults.[12–17]

The human gut is a host to a diverse and dynamic community of microbes that encode proteins not found in the human genome and that play numerous diverse roles in metabolism and energy homeostasis. Current research suggests that disruptions in the normal balance of gut microbial populations are linked to a variety of gut-related disease and conditions, such as metabolic syndrome and obesity.[18,22,23] Although what constitutes a "healthy" gut microbiome remains unknown, it is clear that diversity and abundance of microbial populations is essential. A decrease in microbial diversity have been demonstrated in multiple disease states, including obesity, inflammatory bowel disease, T2D, and colorectal cancer.[18,25,26,61]

Significant and meaningful changes in the gut microbiota have been associated with dietary alterations, primarily consumption of dietary fiber, fat, sugar, and being breastfed during infancy.[25–27,61] However, the mechanism and magnitude of dietary intake influence on the composition of the gut microbiome is not clear. No study has examined the relationship between the microbiome and diet exclusively in a high-risk Hispanic young adult (ages 18-19) population. In addition, although breakfast composition has

been explored[73,135–137] no study has examined the effect of breakfast intake versus skipping breakfast, nor examined the influence of eating frequency on the gut microbiome. Given that Hispanics are disproportionately affected by obesity and metabolic disease and college years are a critical transition period in which lifetime eating habits are established, understanding how dietary intake impacts the gut microbiome in this population is warranted. Thus, the **overall goal** of this study is to examine the relationship between diet and the gut microbiome in Hispanic college freshmen.

SUBJECTS AND METHODS

Participants

Figure 5.1 provides a detailed flow of study participants. The original purpose of this study was to examine the relationship between eating frequency and adiposity and metabolic markers. Hispanic college freshmen subjects were recruited via announcement in classes, word of mouth, electronic posted notices, and tabling at dorms around the UT-Austin campus. Subjects completed a screener to determine eligibility. Inclusion criteria included: (i) self-report that all four of their grandparents were of Hispanic origin (ii) 18-19 years of age, and (iii) in their first year of college. Exclusion criteria included (i) current pregnancy, (ii) taking any medication known to affect body composition or any psychoactive medication, (iii) diagnosis with a disease(s) or syndrome known to affect body composition or fat distribution, (iv) if they had a learning impairment(s) that would complicate survey administration, (v) braces, a pacemaker, or any other contraindications to magnetic resonance imaging scanning, or (vi) participation in a weight loss, dietary, or

physical intervention in the previous six months. Of the 791 eligible Hispanic students, dietary recalls were conducted in 100 subjects. Seventy-nine of those subjects contributed a fecal sample, but 76 were analyzed due to unreadable labels on two of the samples and one subject having an extremely low carbohydrate intake of less than 5% of total daily intake.

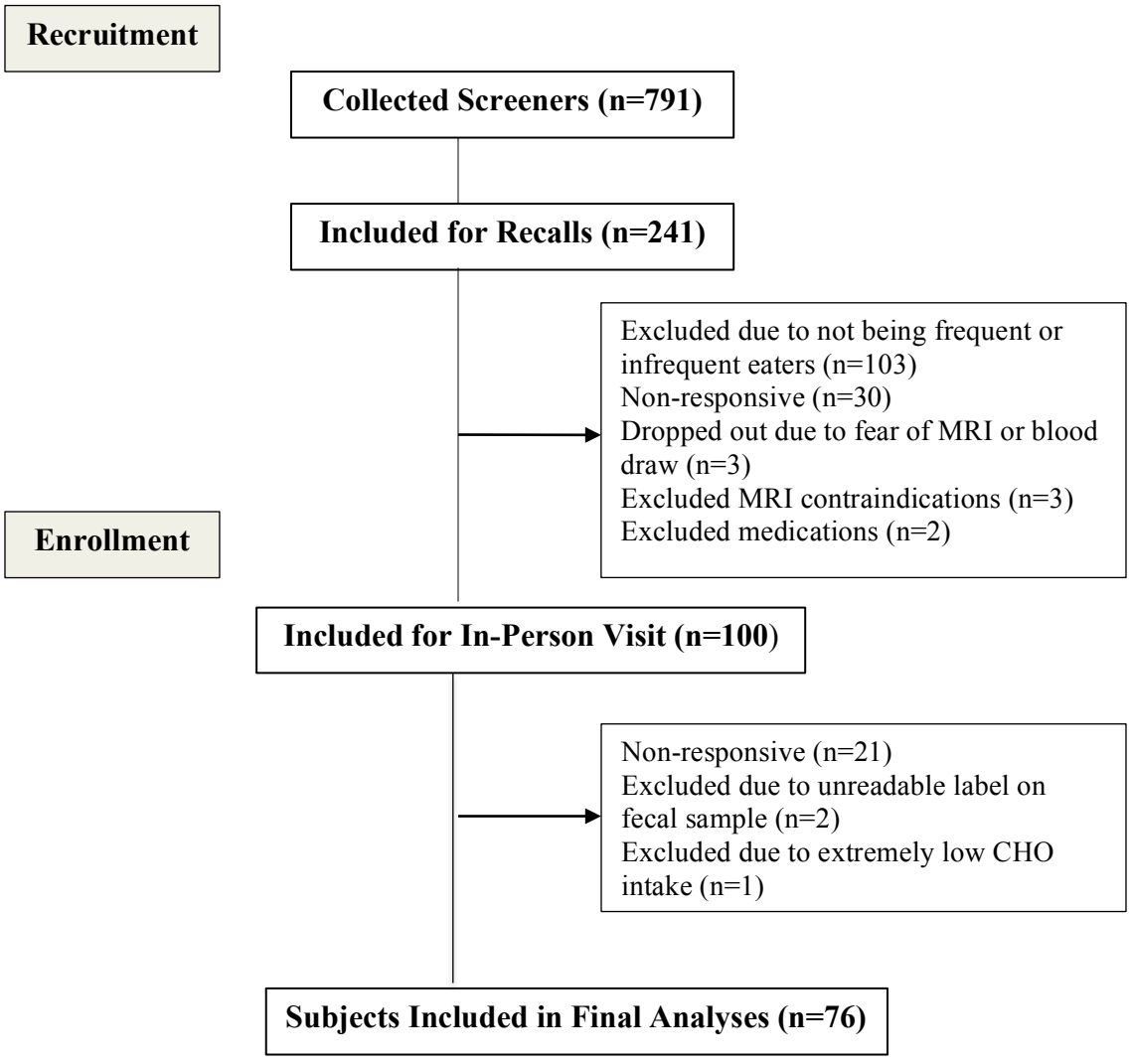

Figure 5.1: Recruitment and enrollment of study participants

Dietary Intake

Dietary intake was assessed from at least three 24-h diet recalls (one weekend and two weekdays) using the multiple-pass technique. Research staff collecting the diet recalls were trained and supervised by a Registered Dietitian. On average, recalls were administered within five days from the in-person testing visit. All dietary recall data were double-entered by trained research staff. Nutritional data was analyzed by using the Nutrition Data System for Research (NDS-R, 2014). The NDS-R program was used to calculate key dietary variables for this analysis, including mean energy intake, total fat, protein, carbohydrates, saturated fat, total sugar, added sugar, dietary fiber, soluble fiber, and insoluble fiber. Prospectively, no recall was performed if the subject indicated being ill. Plausibility of energy intake was assessed by regressing caloric intake against body mass index, and no subjects were over two standard deviations from the mean (n=99).

Stool Samples

Participants were given a kit for stool sample collection with instructions and a brief questionnaire regarding past gastrointestinal illness, antiobiotic use, and supplement use within one week of their in-person visit. Participants were instructed to collect the first stool of the day with a pre-moistened sample wipe, which was stored in a labeled biosafety bag and picked up by research staff within 24-hours of collection. Seventy-nine subjects returned a stool sample. Two of those subjects were excluded due to unreadable identifiers on the stool sample (n=77). Fecal DNA was stored in a -80 freezer and extracted by trained research staff using the Fecal DNA Isolation Kit (Zymo Research,

cat. no. D6010. Coded specimen was then shipped to the Microbiome Resource Laboratory to undergo 16S ribosomal RNA gene sequencing.[66]

The DNA was amplified via polymerase chain reaction (PCR) using degenerate primers flanking the V4 region of the rRNA gene to generate a 250 base pair amplicon. The individual samples were electrophoresed on agarose gel and visualed by UV illumination. The PCR product was excised and purified using a commercial (QIA) gel extraction kit (Qiagen, cat. No. 28704). The purified PCR products were then quantitated using Pico Green dsDNA reagent then sequenced using the NextGen sequencing Illumina Miseq platform from both the 5' prime and the 3' end. [66]

Microbial Composition Analysis

Fecal microbiome composition was analyzed in the microbiome analysis package Quantitative Insights Into Microbial Ecology (QIIME) v1.9.0 and simplified with QWRAP, an online statistical software tool to test differences in the microbe composition within groups.[66] Standard methods for quality control, generating abundance, descriptive statistics of sample bacteria, significant differences between groups, and the specific bacteria between the groups contributing to those differences.[64] Within QWRAP, a quality control check was done using FASTQC v0.11.2 and FASTX v0.0.13 in which all of the raw data are trimmed to reads with over at least 80% base-pairs retained.[66] Counts per sample ranged from 12,700 to 187,656. Clusters of reads with sequence similarity above a 97% cutoff were binned into Operational Taxonomic Units (OTUs), which were counted and used to measure relative abundance.[64,66] Rarefaction curves were generated

to ensure sufficient depth and measure alpha diversity.[64,66] Nonparametric two-sample t-tests compared the alpha diversities between two groups with Monte Carlo permutations.[66] A p-value of < 0.05 was used to determine significance. Unweighted UniFrac, weighted UniFrac, and Bray-Curtis analysis was used to estimate beta diversity, which calculates distance as a measure of similarity of microbial communities between samples or groups of samples.[66] Kruskal-Wallis tests were used to identify differential abundant bacterial phylotypes. A p-value of <0.05 with a false discovery rate (FDR) of <0.2, as was used in the Human Microbiome Project (HMP),[19] determined significance in order to adjust for multiple hypothesis tests.[107] Microbiome composition was then examined for differences between diet groups.

Dietary Groupings

Diet variables were also grouped into tertiles using SPSS version 20.0 (SPSS, Chicago, IL). Diet variables were also grouped according to current dietary recommendations.[83] Values for each participant were classified as "Met" or "Exceeded" dietary recommendations. Participants were classified as meeting recommendations for macronutrient and sugar categories if 45-65% of total daily kilocalories were composed of carbohydrates, 25-35% of total daily kilocalories were composed of fats, 10-30% of total daily kilocalories for protein, or <15% of total daily kilocalories were composed of added sugar.[83,95] For saturated fat, participants were classified as meeting recommendations if <10% of total daily kilocalories were composed of saturated fat. For dietary fiber, men with ≥38 grams of fiber/day or females with ≥25 grams of fiber/day

were classified as meeting recommendations.[83,97] If a participant fell outside of the guidelines, he/she was classified as below or above recommendations. Breakfast eaters waere defined as participants who consumed foods that constituted ≥15% of total daily energy within three hours of waking.[138] Those who ate breakfast on all three days of dietary assessments were considered ALWAYS breakfast consumers, while those who consumed breakfast on one or two days of the three days were defined as INTERMITTENT breakfast consumers, and those who never consumed breakfast were defined as NEVER breakfast consumers. Eating occasions (EO) were defined as ≥50 kilocalories and ≥15 minutes from any previous EO.[139] Participants who had on average four or more EO per day were classified as FREQUENT eaters and those who consumed on average less than three EO per day were classified as INFREQUENT eaters. The classifications were performed separately by two different researchers and compared for reliability.

RESULTS

Participant Characteristics

Demographics and dietary intake are displayed in **Table 5.1**. The average age of participants was 18 years old, 55% were female, and 25% were overweight or obese. Fifty percent of participants exceeded recommendations for total fat and 62% exceeded recommendations for saturated fat. Ninety-seven percent of participants met protein recommendations and 66% met carbohydrate recommendations. Twenty-five percent met added sugar recommendations, but most consumed less than the recommended limit,

whereas only 5% met fiber recommendations.

Table 5.1: Demographics and diet of study participants. Data presented in mean ±SD or n (%).

Subject Characteristics (n=76)	
Sex M/F	34/42
Age (y)	18.7 ±0.4
BMI	23.6 ±3.7
Overweight or Obese	19 (25)
Dietary Variables (n=76)	
Energy intake (kcal/d)	1973.6 ±732.7
Fat (% daily kcal)	34.3 ±5.6
Saturated Fat (% daily kcal)	10.8 ±2.5
Protein (% daily kcal)	17.7 ±4.7
Carbohydrate (% daily kcal)	47.8 ±7.5
Added Sugar (% daily kcal)	3.3 ±1.8
Total Fiber (g)	17.2 ±7.5

Characterizing the Hispanic gut microbiome

Figure 5.2 shows Hispanic freshmen college students have microbiomes primarily composed of 57.2% *Firmicutes*, 33.2% *Bacteroidetes*, 5.3% *Actinobacteria*, 3.3% *Proteobacteria*, 0.5% *Verrumicrobia*, and 0.01% *Other*. Compared to HMP, a healthy population of Americans, and MetaHIT, a healthy population of Europeans,[69] FHS had percentages of bacteria that fall within the range of the two healthy populations with the exception of *Actinobacteria* which had more relative abundance than HMP and MetaHIT (5% vs. 0% and 2%, respectively).

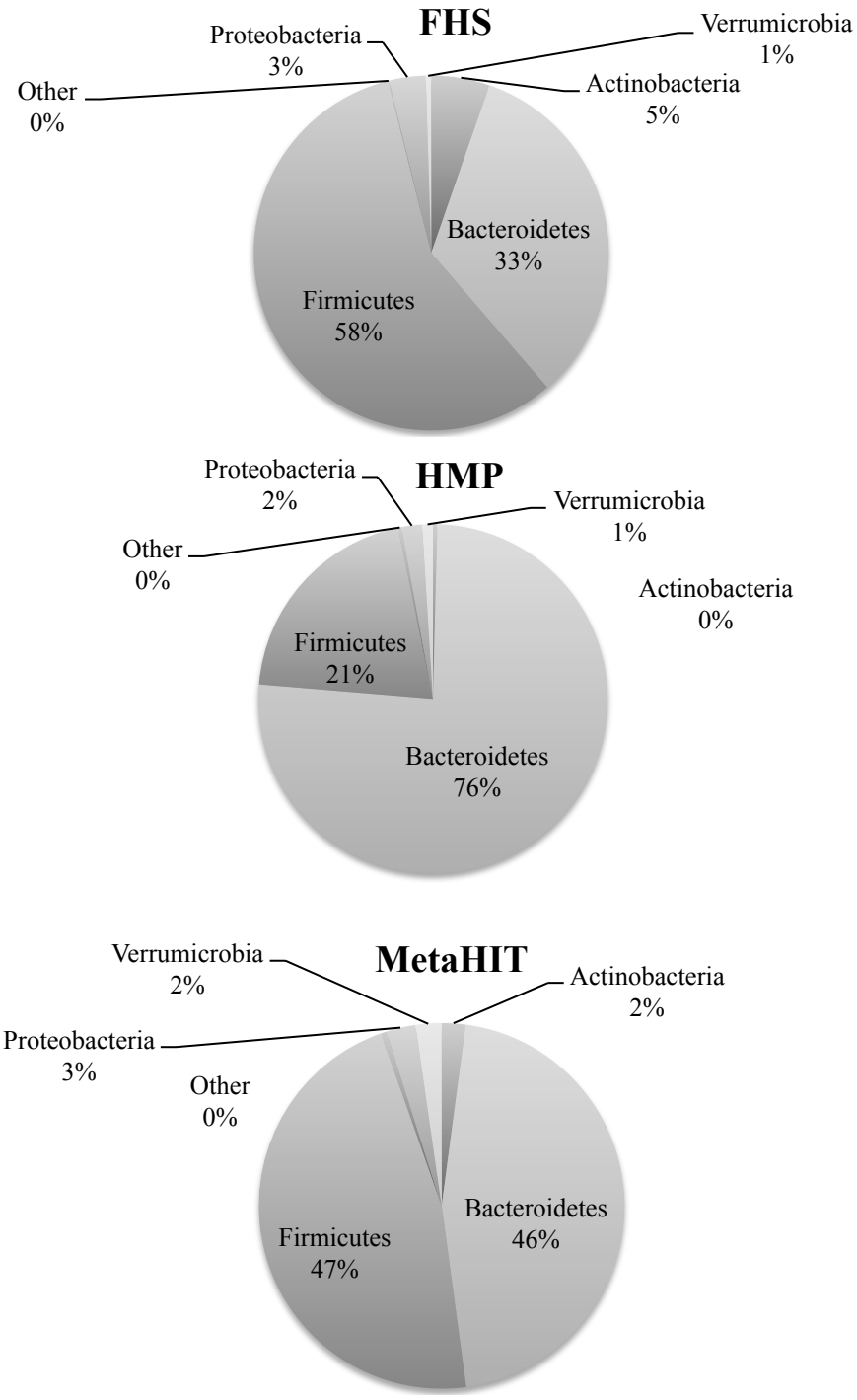

Figure 5.2: Quantitative comparison of relative taxonomic abundance of fecal microbiota in the Freshmen Health Study compared to HMP and MetaHIT.

Comparing beta-diversity by tertiles and by recommendations, saturated fat was the only dietary variable with significant differences, thus the data is only presented using saturated fat recommendations (i.e., those who met vs. those who exceeded recommendations). Compared to subjects who met dietary recommendations to those who did not, saturated fat was the only dietary variable with significant beta-diversity differences (un-weighted p=0.01) as seen in **Table 5.2**. Bray-Curtis and weighted p-values did not achieve significance. The gut microbiome was not significantly different in other dietary variables, such as subjects who met recommendations for carbohydrates, total fat, protein, total fiber, insoluble fiber, soluble fiber, total sugar, added sugar. In addition, breakfast intake and eating frequency were not significantly linked to the gut microbiome. When dietary variables were grouped according to tertiles, saturated fat was again the only dietary variable contributing a significant difference (p=0.00) in microbiome composition (data not shown). Tertiles of carbohydrates, total fat, protein, total fiber, insoluble fiber, soluble fiber, total sugar, added sugar were not significantly linked to the gut microbiome. **Figure 5.3** shows the Shannon biodiversity index of increased diversity of the microbiome in subjects who met saturated fat recommendations compared to those who exceeded recommendations (5.21 ±0.90 vs. 4.92 ±0.52; p=0.01).

Table 5.2: Beta-diversity between groups of recommendations of dietary variables. Category data presented in n (%); p-value is unweighted

Dietary Nutrients	**Categories**			**β-diversity p-value**
	Below	*Met*	*Exceeded*	
Carbohydrates	26 (34)	50 (66)	0 (0)	0.43
Fat	3 (4)	38 (50)	35 (46)	0.78
Protein	0 (0)	74 (97)	2 (3)	0.60
Added Sugar	64 (84)	12 (16)	0 (0)	0.77
Saturated Fat	0 (0)	29 (38)	47 (62)	0.01*
Total Fiber	72 (95)	4 (5)	0 (0)	0.12
Dietary Patterns	**Categories**			**β-diversity p-value**
Breakfast Consumption	*Never*	*Intermittent*	*Always*	
	17 (22)	45 (59)	14 (18)	0.66
Eating Frequency	*Infrequent*		*Frequent*	
	34 (45)		42 (55)	0.52

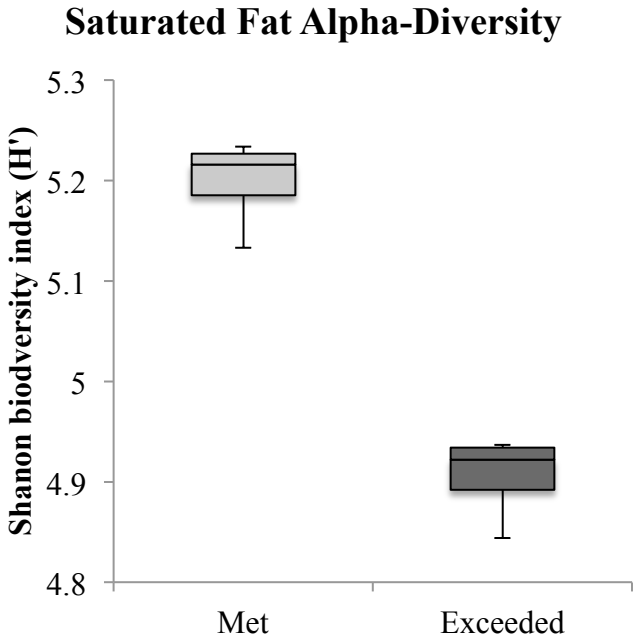

Figure 5.3: Microbiome biodiversity between subjects who met and exceeded saturated fat recommendations. Those who exceeded saturated fat recommendations compared to those who met them had less diversity in the human gut microbiome (overall biodiversity 4.92 ±0.52 vs. 5.21 ±0.90; p=0.01)

Participants who exceeded saturated fat recommendations compared to participants who met saturated fat recommendations had significantly less relative abundance of order *Victivallales* (1.4 x 10^4 vs. 1.0 x 10^5; p=0.04, FDR 0.18) member of the *Lentisphaeria* class and *Lentisphaerae* phylum, order *Methanobacteriales* (5.3 x 10^4 vs. 9.0 x 10^5p=0.03, FDR 18%) member of the *Methanobacteria* class and *Euryarchaeota* phylum, order *Campylobacterales* (7.5 x 10^3 vs. 2.7 x 10^3; p=0.04, FDR 18%) member of the *Epsiloprotebacteria* class, and order *Bacillales* (3.8 x 10^3 vs. 1.2 x 10^3; p=0.04, FDR 18%) while linked to inverse effects in order *Other* as seen in **Figure 5.4.** Genus and

species did not achieve significance in any of the bacteria.

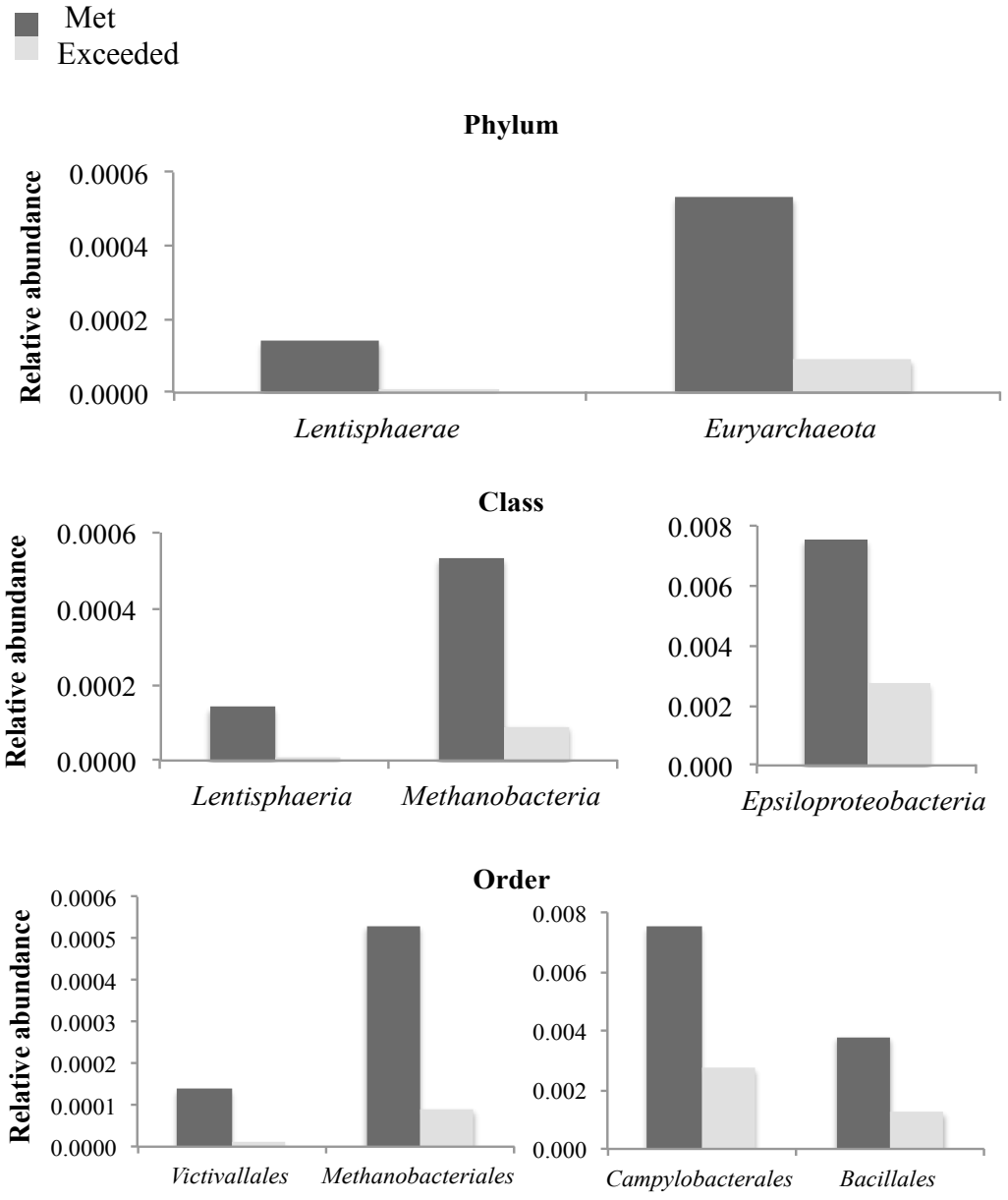

Figure 5.4: Relative abundance of significant bacteria between participants who met and exceeded saturated fat recommendations. Data presented in mean relative abundance, with $p < 0.05$ and FDR < 0.2.

DISCUSSION

This is the first study to examine the relationship between dietary intake and the microbiome in an exclusively Hispanic college population. *Firmicutes* were the dominant bacteria found in this population. Saturated fat was the main dietary variable contributing to an altered microbiome. Those who exceeded saturated fat recommendations compared to those who met it had lower microbial composition diversity, reflected by a decreased relative abundance of *Victivallales, Methanobacteriales, Campylobacterales*, and *Bacillales*.

Decreases in microbial biodiversity are linked to metabolic disease states and a more diverse microbiome is linked to a healthy profile.[140] In a study comparing mice fed a normal chow diet to mice fed a high fat diet, mice consuming a high fat diet had significantly lower diversity than their normal chow fed counterparts.[141] In humans, a study conducted between children living in Burkino Faso who typically consume high-fiber diets, compared to Italian children who typically consume a Western Diet, found that the Western diet group had significantly lower richness and biodiversity than the high-fiber group.[25] Our study confirms these findings in an exclusively Hispanic population, adding that specifically saturated fat is linked to a less diverse microbiome.

A higher abundance of *Firmicutes* in the microbiome has been linked to unhealthy diets and metabolic profiles.[140] Multiple studies in mice comparing standard chow to mice put on a high fat diet consistently showed significantly decreased counts of *Bacteroidetes* and increased amounts of *Firmicutes* and *Proteobacteria*.[30,141] In humans, the study done between children living in Burkino Faso and Italy found diets higher in fat to have higher

counts of *Firmicutes*.[25] HMP is one of the few ongoing human studies in the United States that showed that a diet high in fat and low in dietary fiber was positively linked to phylum-level *Bacteroidetes* and *Actinobacteria* and inversely linked with *Firmicutes* and *Proteobacteria*.[74] On the phylum level, like HMP, the current study found types of *Firmicutes* (*Bacillales*) and *Proteobacteria* (*Campylobacterales*) to be significantly less abundant in the participants who exceeded saturated fat recommendations. Unlike HMP, this study did not find any other dietary variable, specifically dietary fiber, to be linked to the gut microbiome. It is important to note that there are several differences in methodology between the HMP and the current study, including the HMP used food-frequency questionnaires to assess dietary intake, ran Spearmen correlations, and did not report on the order-level of the bacteria, therefore the results are not directly comparable. However, the current findings support these studies done in mice and humans that diets high in saturated fat are linked to an altered microbiome.[30]

This study was the first to examine breakfast eating vs. breakfast skipping and meal frequency. Although decreased eating frequency has been linked to increased visceral adipose tissue, body fat, and obesity risk in this population,[16,142] eating frequency was not shown to affect the microbiome. Multiple studies have confirmed that a whole-grain breakfast contributes to a prebiotic effect to the human gut microbiota.[73,136,137] However, breakfast skipping which has been associated with visceral fat and insulin indices in overweight Latino youth,[15] was not significantly related to the gut microbiome. This suggests that in this cross-sectional study, nutrient content rather than nutrient timing contributed to the composition of the human gut microbiome. Longitudinal and

intervention studies are needed to assess the impact of nutrient timing on the gut microbiome.

We found increases in the relative abundance of microbiomes that have been associated with the breakdown of fiber. *Victivallales* has been detected in cows and aids in vegetal fiber degredation.[143] *Methanobacteriales* uses H_2 to reduce CO_2 to CH_4, which is produced exclusively through breaking down carbohydrates in humans. [144,145] When we examined the microbiomes of those who met fiber recommendations and those who fell below, there were no significant differences. However, subjects who exceeded fat intake versus those that met them had significantly lower intakes of dietary insoluble fiber (13.41 ±0.95 grams/day vs 10.74 ±0.74 grams/day; p=0.04). These findings suggest that diets high in saturated fat lack bacteria that break down fiber. This could possibly be due to bacteria that break down fat competing with bacteria that break down fiber, or that the population in our study had a fairly homogenous diet regarding fiber.

Two other bacteria found to be significantly less abundant in those who exceeded saturated fat recommendations were *Campylobacteria* and *Bacillales*. *Campylobacteria* is recognized as bacteria associated with diarrhea, however its role or proof of being a pathogen has not been proven. [132] *Bacillales* are known to produce antimicrobial compounds, which function as an antibacterial or antifungal and used to treat or prevent infection.[146] The specific functions of these bacteria in humans remain elusive and further examination would be important.

The limitations of this study include the cross-sectional design and the relatively small sample size. This population was homogenous in ethnicity, location, and age,

eliminating possible confounders. However, in some instances the diet was homogenous as well such as protein recommendations being met and fiber recommendations not being met by nearly the entire sample. A larger sample size, a more diverse diet, and deeper amplification are needed to further explore significant differences in specifying bacteria available in smaller amounts.

In summary, our study is consistent with other studies that have reported that subjects with diets high in saturated fat have a less diverse microbiome. Saturated fat has been routinely linked to cardiometabolic diseases[82,105,141] and a decrease in microbial diversity has been linked to metabolic diseases such as obesity and T2D.[24] The effect of saturated fat on the microbiome could be one mechanism underlying these diseases. Although diet may be playing a role in cardiometabolic disease etiology, it may be a small role and more exploration is needed to confirm the potential mechanism.

Few studies have implicated human consumption of fat on the overall decreased diversity of the microbiome and order-level identification of specific bacteria. Metabolic diseases disproportionally affect Hispanics[11] and no studies have examined the effects of diet on the microbiome in an exclusively Hispanic college population. This study suggests that a decrease in saturated fat intake is recommended in order to increase biodiversity and could have downstream impacts on decreases in cardiometabolic and/or inflammatory diseases. More longitudinal and intervention studies are warranted to understand the role that diet plays on the gut microbiome and subsequent disease risk.

Chapter 6: Conclusion

The purpose of this research was to study the relationship between diet, gut microbiome, and disease risk in a vulnerable and understudied population of Hispanic college freshmen. Specifically, this dissertation characterized the young adult Hispanic gut microbiome and examined the relationship between 1) diet and eating patterns with adiposity and metabolic measures, 2) adiposity and metabolic measures with the gut microbiome, and 3) the gut microbiome and diet and eating patterns. This cross-sectional study of college Hispanic freshmen (18-19 years of age) revealed that total dietary fat and saturated fat were positively linked to SAT, total body fat, insulin, insulin resistance, leptin, and CRP, and dietary saturated fat was linked to hepatic fat. Furthermore, the odds of having NAFLD increased by 34% for every percent increase of dietary saturated fat. Carbohydrates were positively linked to CRP, total sugar was positively linked to triglycerides, and added sugar was positively linked to VAT. On the other hand, fiber was inversely linked to hepatic fat, glucose, insulin, insulin resistance, and leptin. These results support existing literature that high intakes of dietary saturated fat and low intakes of dietary fiber are linked to obesity and related diseases.

Saturated fat was also the only significant variable linked to the gut microbiome. Those who met saturated fat recommendations had significantly higher biodiversity compared to those who exceeded saturated fat recommendations. Upon further analysis, those who exceeded saturated fat had decreased relative abundance of the potentially beneficial bacteria: *Victivallales, Methanobacteriales, Campylobacterales*, and *Bacillales*. *Victivallales* and *Methanobacteriales* participate in carbohydrate breakdown;

Campylobacterales have an unknown function; and *Bacillales* upregulates antibacterial and antifungal compounds. [132,143–145] However, when analyzing the gut microbiome in subjects with and without NAFLD, overall diversity was not significantly different. This suggests that the gut microbiome may not explain the link between saturated fat and NAFLD seen in Chapter 3.

When the gut microbiome was analyzed by tertiles of adiposity and metabolic measures, total percent body fat, insulin, and LDL were the only significant variables. Subjects with high percent body fat and insulin compared to those with low and moderate percent body fat and insulin had lower microbial composition diversity. On the contrary, participants with low and moderate LDL levels compared to subjects who had high levels of LDL had lower microbial composition diversity. Subjects with high percent body fat had lower relative abundance of *Methanobrevibacter, Akkermansia*, and *Clostridiales*. *Methanobrevibacter* and *Akkermansia* have been correlated with leanness, although exact function is unknown, and *Clostridiales* participates in degradation of fiber. Subjects with high insulin had lower relative abundance of *Proteobacteria*. However, no conclusions can be made without further specificity of the bacteria species within this very diverse phylum. Low values of LDL had decreased relative abundance of *Jonquetella anthropi, Actinomyces europaeus, Pyramidobacter, Peptococcus, Moryella, Mobilincus,Campylobacter, Facklamia, Mogibacterium, Gallicola, WAL_1855D,* and *I-68*, most of which do not have designated functions. *Jonquetella anthropic, Actinomyces europaeus*, and *Pyramidobacter* aid in the production of short chain fatty acids, which decrease inflammation. [61,125–128] *Peptococcus* and *Moryella* are involved in various forms

of transportation and signaling to aid in the function of LDL. [129,130] These results confirm that the gut bacteria that are present in leaner subjects participate in fiber digestion. However, the LDL findings were contrary to the hypothesis. Upon further inspection, the range of LDL values fell within the recommended range; meaning subjects who had lower amounts of LDL had insufficient stores of LDL compared to those who had optimal levels of LDL. In addition, the specific bacteria found in those who had optimal levels compared to those who had suboptimal levels were bacteria that aided in LDL function. This provides support that LDL is beneficial to the body at certain ranges and that the microbiome is a mechanism for blood lipids. Therefore, the gut microbiome may be a mediating factor involved in cardiovascular disease risk.

Although there were many significant results, there were also many null results. Eating patterns such as frequency of meals and breakfast did not achieve significance; suggesting nutrient content may be a stronger driver of the microbiome than nutrient timing. However, intervention and longitudinal studies are needed to assess the true impact of nutrient timing. Tertiles of dietary variables did not achieve significance in microbiome diversity between subjects, nor did adiposity and metabolic measures split into healthy versus unhealthy ranges. This is most likely due to the homogeneity of the sample, and in some instances the diet. Having a sample of the same age, ethnicity, and location limited confounders. However, this also inadvertently created bias. This group did not provide enough unhealthy subjects to analyze overall disease risk. These Hispanic college freshmen were leaner, more active, and more metabolically healthy than the general Hispanic adult population, although their diets were similar to the national

diet averages.[97,103] However, having a homogenous sample was ideal for characterizing a health emerging young adult Hispanic microbiome.

The young adult Hispanic microbiome was predominantly made up of the phylum *Firmicutes*, followed by *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrumicrobia*. Figure 5.2 shows how the gut microbiome of Hispanic youth matches up to healthy adult populations of differing ages (18 years of age and up) and ethnicities in both Europe (MetaHit) and the US (HMP). [69] The Hispanic microbiome is more similar to those subjects in Europe. In addition, when comparing the Hispanic microbiome to children (1-6 years of age) in Italy and in Burkino Faso, the young adult Hispanic microbiome is more similar to the Italian children than it is to the HMP, despite being a US population.[25] This suggests that the microbiome of college students may be more similar to a child microbiome than an adult microbiome and that age may be a more defining factor than location. Population wide samples should control for age and ethnicities when characterizing a healthy human gut microbiome.

In conclusion, this was the first study to examine the relationship between dietary intake, adiposity, metabolic parameters, and the gut microbiome in a Hispanic college population. It is also the first study to directly examine the relationship between diet, metabolic diseases, and the gut microbiome in an exclusive Hispanic population. Findings support previous studies that diets high in fiber compared to those low in fiber are linked to lower adiposity, healthier metabolic outcomes, and more diverse microbiomes, specifically bacteria that breakdown fiber. [49,81,143] On the other hand, diets high in saturated fat compared to those low in saturated fat have higher adiposity and

decreased microbiome biodiversity, specifically a reduction in metabolically beneficial bacteria[25,74,141] Saturated fat exhibited a strong relationship with the incidence of NAFLD, like that found in overweight and obese women in another study.[89] However, while the gut microbiome may still be a potential mechanism between saturated fat and NAFLD, in this study, the microbiome did not significantly modulate the relationship. This finding reinforced previous studies that reported no significant difference between subjects with and without NAFLD.[42,43] This could be due to the number of NAFLD subjects and should be studied in a larger sample and more directly. A saturated fat intervention in NAFLD patients would be a better design for determining the mechanism involving the microbiome on diet and disease. On the contrary, LDL was shown to have a relationship with the gut microbiome and HDL was approaching significance, attesting to the gut microbiome being the potential mechanism behind cardiovascular diseases.

These results collectively indicate that this population would benefit from an intervention targeting saturated fat. These participants were not sugar consumers as suspected with an average of 70.5 grams/day in comparison to the national average in a similar age group of 105 grams/day,[147] which suggests that tailored interventions for different populations are warranted. In this case, Hispanic college freshmen consumed high amounts of saturated fat, which correlated significantly with the microbiome. Comparing baseline microbiome bacteria diversity and abundance over time pre and post saturated fat intervention would explore a potential cause and effect relationship between saturated fat and microbiome diversity. A suggested intervention of one year or longer would be useful to illustrate any lasting effects. This intervention would be particularly

interesting in subjects with NAFLD in order to directly study any impacts being made in disease progression via diet and microbiome. In addition, despite the amounts of fiber being consumed below recommended amounts, there was a relationship between fiber, adiposity, and some metabolic measures. Further research on a range of fiber intake, as well as a possible dietary intervention replacing saturated fat with fiber, would be beneficial for studying dietary mechanisms and disease prevention.

Decreases in microbial biodiversity are linked to metabolic disease states that plague the Hispanic population such as obesity, diabetes, NAFLD, and cardiovascular disease. This study highlights that diet is linked to the gut microbiome and may be a potential mechanism explaining how diet impacts obesity and its accompanying morbidities. These findings support other studies that show that diets high in dietary fiber and low in saturated fat are linked to low levels of adiposity, metabolic disease risk, and healthier microbiome profiles as predicted in the hypothesis. More longitudinal and intervention studies are needed to better understand these relationships.

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Vita

Education

Dates	*Degree*	*Institution*
2011- present	Seeking PhD	The University of Texas at Austin *Department of Nutritional Sciences*
2012-present	Seeking Master of Science	The University of Texas at Austin *Department of Statistics and Data Science*
2011-2015	Master of Arts	The University of Texas at Austin *Department of Nutritional Sciences*
2007-2011	Bachelor of Science	The University of Texas at Austin *Department of Kinesiology*

Research and Professional Experience

2012- 2017	The University of Texas at Austin *Department of Nutritional Sciences*	Teaching assistant for NTR 306 *Dr. Jeanne Freeland-Graves*
2017	The University of Texas at Austin *Department of Nutritional Sciences*	Teaching assistant for NTR 337 *Dr. Ladia Hernandez*
2015-2016	The University of Texas at Austin *Department of Nutritional Sciences*	Teaching assistant for NTR 338W *Dr. Ladia Hernandez*
2016	The University of Texas at Austin *Department of Nutritional Sciences*	Teaching assistant for NTR 307 *Mrs. Drew Hays*
2016	The University of Texas at Austin *Department of Nutritional Sciences*	Teaching assistant for NTR 126L *Dr. Alessia Ladi*
2014-2015	The University of Texas at Austin	Teaching assistant for NTR 118L *Dr. Monica Meadows*
2014	The University of Texas at Austin	Teaching assistant for NTR 218 *Dr. Monica Meadows*
2013	The University of Texas at Austin *Department of Nutritional Sciences*	Study Coordinator for the Exploring Health and Lifestyle in Firefighters Study *Dr. Michele Forman*
2012	The University of Texas at Austin *Department of Nutritional Sciences*	Lead research assistant for The National Children's Study-Pilot, Travis

		County *Dr. Michele Forman*
2012	The University of Texas at Austin *Department of Nutritional Sciences*	Teaching assistant for NTR 306 *Dr. Jeanne Freeland-Graves*
2011-2015	The University of Texas at Austin *Department of Nutritional Sciences*	Graduate assistant for The Nurses's Mothers Cohort Study *Dr. Michele Forman*
2011	The University of Texas at Austin *Department of Nutritional Sciences*	Graduate assistant for Provider Based Sampling Study for The National Children's Study, Harris County *Dr. Michele Forman*
2011-2015	The University of Texas at Austin *Department of Nutritional Sciences*	Teaching assistant for NTR 112L *Mrs. Lydia Steinman*

Certifications

2007-present	Certified Athletic Trainer, Licensed Athletic Trainer

Professional societies

American Society of Nutrition	Student Member

Title of Dissertation	The associations between nutrient intake and eating patterns with adiposity, metabolic risk factors, and the gut microbiome in Hispanic college freshmen
Dissertation Advisor	Jaimie N Davis, PhD, RD University of Texas at Austin School of Human Ecology Department of Nutritional Sciences

Manuscripts in Review

Pilles KN, Van Der Pol WJ, Morrow CD, Asigbee FM, Davis, JN. *Altered composition of fecal microbiome associated with body fat, LDL, and insulin in Hispanic college students* (In preparation for American Journal of Clinical Nutrition).

Manuscripts in Preparation

Pilles KN, Van Der Pol WJ, Morrow CD, Asigbee FM, Davis, JN. *Altered composition of fecal microbiome associated with body fat, LDL, and insulin in Hispanic college students* (In preparation for American Journal of Clinical Nutrition).

Pilles KN, Van Der Pol WJ, Morrow CD, Asigbee FM, Bray MS, Davis, JN. Saturated fat intake correlates with altered composition of fecal microbiome in Hispanic college students (In Preparation for American Journal of Clinical Nutrition).

Asigbee FM, **Pilles KN**, Davis JN. How high school sports participation plays a role in college physical activity. (In review at Medicine & Science in Sports and Exercise)

Markowitz AK, Landry MJ, **Pilles KN**, Khazaee E, Ghaddar R, Vandyousefi S, Asigbee FM, Gatto NM, Spruijt-Metz D, Davis JN. *Positive association between cooking and gardening behaviors and improvements in dietary intake in Hispanic youth.* (In review at American Nutrition and Dietetics Journal).

Landry MJ, Khazaee E, Markowitz AK, Vandyousefi S, Ghaddar R, **Pilles KN**, Asigbee FM, Gatto NM, Davis JN. *Food Security and Glycemic Control Among Low-Income Hispanic Children in Los Angeles, California.* (In review at Public Health Nutrition).

Awards and Honors

2015	Dr. John B. Longenecker Graduate Research Summer Award
2014	Summer Fellowship Award in Nutrition
2012	Certificate of Recognition and Appreciation for Outstanding Service and Dedication to The National Children's Study – Formative Research Project "Evaluation of Ulnar Length Measurement for Use in the NCS"

Conference Poster Presentation

Experimental Biology	**Pilles KN,** Dong Y, Michels KB, Willett WC, Forman MR. *Filling the gap: trends in and factors related to infant feeding choice in the U.S. from 1925-1964*

April 22, 2013
Boston, MA

The Obesity Society November 4, 2016 *New Orleans, LA*	**Pilles KN**, House BT, Shearrer GE, Markowitz AK, Asigbee FM, Davis JN. The link between *dietary intake and adiposity and metabolic parameters in Hispanic college students*

Seminars, Lectures, Orals

University of Texas April 27, 2017	**The Gut Microbiome** NTR 365: Obesity and Metabolic Health
University of Texas November 15, 2016	**The Gut Microbiome** NTR 365: Obesity and Metabolic Health

NAFLD

Students with NAFLD had a significantly higher intakes of saturated fat (11.89 ±0.71 % kcals versus 10.12 ±0.25 % of kcals , $p<0.001$) as seen in **Figure 2.2**. The odds of having NAFLD increase by 34% (95% CI: 1.08, 1.65, p=0.04) for every percent increase of dietary saturated fat.

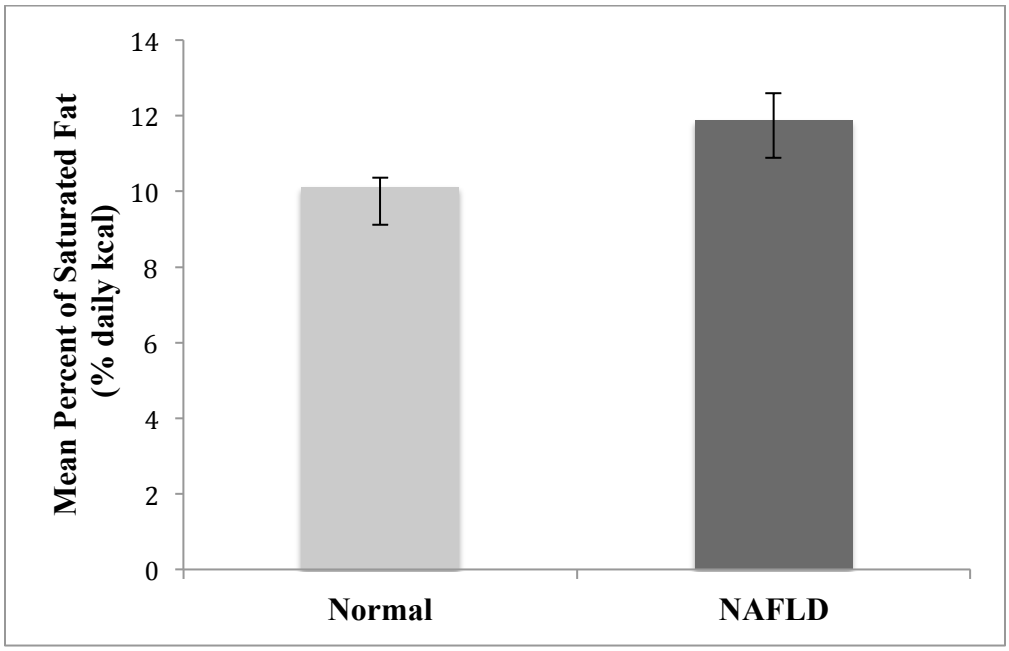

Figure 2.2: Dietary saturated fat intake in subjects with non-alcoholic fatty liver disease (NAFLD) was higher compared to normal subjects. Data presented as mean ±SD.

DISCUSSION

This is the first study to examine the relationship between dietary intake and adiposity and metabolic parameters in a Hispanic college population. These findings

support previous findings[49,81] that diets high in fiber are linked to lower adiposity and healthier metabolic outcomes while diets high in saturated fat and added sugar are related to higher adiposity and increased risk of metabolic diseases.[12,52,81,82]

Daily dietary fat percentages were high in this population, over 34%, which is near the upper limit of the Dietary Reference Intakes (DRI) recommendation for older children and adults of 25-35%. [83] Saturated fat percentages were above recommended ranges, with 10.6% of daily energy coming from saturated fat compared to the DRI recommendation for children and adults of <10%.[83] These dietary variables were related to increases in SAT, total body fat, insulin, HOMA-IR, leptin, and CRP and may be modulated by the same pathway. A prolonged high-fat diet has been linked to insulin-resistance, thereby requiring an increased production of insulin for glucose absorption.[84] In addition, excess dietary fat increases the size and count of adipocytes that release leptin to regulate body weight and weight gain.[85] Leptin has been shown to up-regulate CRP and indicates an immune response.[86]

Hepatic fat and cholesterol were only increased in the saturated fat variable and implies a specific pathway not associated with unsaturated fats. Furthermore, only dietary saturated fat was linked to increased odds of NAFLD. Previous cross-sectional studies and intervention studies in adults have found that saturated fat contributes to higher levels of adipokines, insulin resistance, and the development of NAFLD.[52,87,88] An intervention study showed that hepatic fat is directly modifiable by diet when a decrease of saturated fat in the diet contributed to a decrease in hepatic fat in overweight and obese women.[89] However, no other study has examined how diet relates to liver fat in a transitional and

high-risk population of college Hispanic freshmen.

The mechanisms behind dietary fat and hepatic fat storage remain unclear, but many theories exist. Mice studies collectively suggest that the liver is the first organ to store excessive amounts of fatty acids since a significant percentage is absorbed by the liver after a meal.[89] A high saturated fat diet may also be correlated with oxidative stress in the liver by impairing glutathione metabolism, and may cause an increased glucose-dependent insulinotropic polypeptide (GIP) response, which is associated with liver disease. [90] Therefore, excessive dietary saturated fat may first be transported to the liver and then oxidized causing an increase in inflammation and fat storage. In addition, CRP is produced by the liver so the increased levels of CRP, leptin, and hepatic fat support this mechanism.[91] However, CRP is produced in response to IL-6 levels, of which were not related to diet in this population.[91] The relationship between IL-6, CRP, and HF suggests that markers other than IL-6 may activate CRP.

On the contrary, students who ate more dietary protein had decreased levels of CRP. The breakdown of amino acids require energy and thereby may increase fat oxidation in the liver and decrease the inflammation markers such as CRP linked to increased levels of fat storage. In addition, derivatives of certain amino acids, such as taurine, may aid in the expression of regulating metabolic genes that decrease inflammation.[90] This is the first study to show that free-living protein intake is linked to lower CRP, and intervention and longitudinal studies should be conducted to further explore this relationship.

We also found that added sugar intake was linked to increased VAT in this population, which is consistent with our previous work in African American and Hispanic, overweight adolescents.[92] Added sugar in the Western diet is increasingly in the form of high fructose corn syrup, which has been shown to specifically up-regulate the expression of lipogenic genes that promote fat storage in VAT.[93,94]

Previously, we found an association between added sugar and insulin resistance in overweight Hispanic youth (ages 10-17 years).[15] However, in the current study no relation between added sugar and glucose/insulin action was found. Possible explanations are that the daily added sugar intake in the current population was fairly low, making up only 282 of daily calories, which is less than the mean added sugar intake in both boys (442 kcals/d) and girls (314 kcals/d) (12-19 years of age) in the US[95], and within the 5%-15% DRI recommended range.[83,95] Another explanation for the null findings is that this population included normal weight subjects, many who were insulin sensitive. Regardless, the rather low added sugar intake was linked to higher VAT.

Carbohydrate intake was linked to increased CRP, which was also seen in an observational study of 244 healthy women.[96] These data suggest that carbohydrates with a high glycemic load contribute to increases in pro-inflammatory processes. However, when isolating carbohydrates to total sugar, we found the only significant increase was in triglycerides, suggesting that carbohydrates may be contributing to excess energy intake that may lead to increased lipid storage that promotes overall inflammation. However, the mechanisms behind these findings need to be further explored.

Dietary fiber intake in this population was relatively low at 16.8 g/d, which is

below the DRIs ranges of 24-35 g/d, although in line with national averages for this age group.[83,97] Dietary fiber in this study was inversely linked to hepatic fat, fasting glucose and insulin, insulin resistance, and leptin. Possible explanations for these findings include phytochemicals within fibrous foods triggering anti-inflammatory pathways.[94] The gel-like properties of fiber may also moderate the glycemic response by slowing down gastric emptying, thereby improving insulin sensitivity and reducing the absorption of macronutrients and storage of hepatic fat.[95] The reduction of anti-inflammatory pathways and also the decrease in fat storage could collectively contribute to the decrease in leptin levels.[98] In addition, there is some evidence that fiber contributes to microbiotic expression of hormones, such as proglucagon derived peptide (GLP-2), which decreases gut permeability and hepatic inflammatory responses.[70] However, further research on the effects of fiber and the microbiome is needed.

Contrary to our previous findings and other studies [81,99–101] dietary fiber was not associated with VAT or inflammatory markers. However, this study differed from the other study populations in age, ethnicity, and weight status. One study was conducted with an older adult population, two studies used Norwegian young adults of varying BMI, and our previous study included both African American and Hispanic adolescents (14-18 years of age) who were all overweight or obese.

There are several limitations to mention. This study was cross-sectional and causality could not be determined. In addition, the sample size was relatively small, these students may be of higher socioeconomic status, and UT-Austin was ranked as a top 20 fittest campus in the US.[102] Our Hispanic young adults appear to be leaner and fitter and

may not be representative of the general Hispanic population as the prevalence of overweight/obesity was only 30% compared to 38% of Hispanic children (2-19 years of age) in the US.[11] However, the diets of this population were similar to the national diet averages.[97,103] As college enrollment in Hispanics continues to rise, it becomes increasingly important to understanding the health consequences of this population.

In conclusion, diets high in saturated fat and added sugar and low in dietary fiber, which is representative of the Western diet, were associated with increases in insulin resistance, inflammatory responses, and increased adipose tissue in specifically HF and VAT, and increased NAFLD in Hispanic college students. This time period is an important transitional period for influencing lifetime dietary habits and determinants of chronic disease. Reductions in dietary sugar and fat and increases in fiber may be potential targets for obesity intervention and prevention efforts in Hispanic populations.

Chapter 4: Altered composition of fecal microbiome associated with adiposity and metabolic parameters in Hispanic college students

Pilles KN, Van Der Pol WJ, Morrow CD, Asigbee FM, Davis JN.

ABSTRACT

Background: Hispanics are disproportionately affected by obesity and its accompanying morbidities such as type-2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and heart disease. All of these disease states have been linked to changes in the composition of the gut microbiome. No study has examined the relationship between the gut microbiome and cardiometabolic risk factors in an exclusively Hispanic young adult (18-19 y) population.

Objective: The purpose of this study is to examine the relationship between cardiometabolic risk markers and the gut microbiome in Hispanic college freshmen.

Design: BMI, waist circumference, body fat via BodPod, hepatic fat (HF), visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT) via magnetic resonance imaging, glucose, insulin, insulin resistance, and lipids via fasting blood draw, and stool samples via fecal wipe were collected in a cross-sectional study of 76 Hispanic college freshmen. Adiposity and metabolic variables were grouped according to tertiles and subjects were classified as having low, moderate, or high values. The microbiome was analyzed for diversity between groups. Significant variables were further analyzed for diversifying bacteria.

Results: Significant differences in microbial diversity were found between tertiles of total body fat, low-density lipoprotein (LDL), and insulin as determined from unweighted

Uni-Frac analysis. Those subjects with high body fat and insulin compared to those with low body fat and insulin had significantly less overall diversity. Those subjects with low values of LDL compared to those with high LDL levels had significantly less overall diversity.

Conclusion: Few human studies have assessed the relationship of adiposity and cardiometabolic risk factors with microbiome diversity, and this is the first study to examine this relationship in an exclusive healthy Hispanic college population. This study suggests that by increasing biodiversity of the microbiome we may thereby decrease inflammatory diseases.

INTRODUCTION

The gut microbiome is a diverse and dynamic community of microbes within the human gastrointestinal (GI) tract whose structure and composition plays a role in metabolism and energy homeostasis.[18] What constitutes a "healthy" gut microbiome remains unknown, but current research suggests diversity of microbial populations is essential. Decreases in microbial diversity have been demonstrated in disease states, including obesity, type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease.[18–20,61,104,105]

Hispanics are disproportionately affected by obesity, T2D, and NAFLD.[11] They are also the largest and fastest growing ethnic minority in the US and in recent history have surpassed Non-Hispanic Whites and Blacks in college enrollment.[1,2] In 2016,

Hispanic students represented 23% of freshman enrollment at the University of Texas at Austin (UT-Austin), having the largest increase among all minority groups.[106]

Given this population is disproportionately affected by obesity and metabolic disease, and that the college years are a critical transition period in which lifestyle habits are established, understanding how adiposity and metabolic markers are linked to the gut microbiome in this population is warranted. Thus, the **overall goal** of this study is to examine the relationship between adiposity and metabolic markers and the gut microbiome in Hispanic college freshmen. Subjects with healthy ranges of adiposity and metabolic markers are hypothesized to have a greater diversity than subjects outside of healthy ranges.

SUBJECTS AND METHODS

Participants

Figure 4.1 provides a detailed flow of study participants. The original purpose of this study was to examine the relationship between eating frequency and adiposity and metabolic markers. Hispanic college freshmen subjects were recruited via announcement in classes, word of mouth, electronic posted notices, and tabling at dorms around the UT-Austin campus. Subjects completed a screener to determine eligibility. Inclusion criteria included: (i) self-report that all four of their grandparents were of Hispanic origin (ii) 18-19 years of age, and (iii) in their first year of college. Exclusion criteria included (i) current pregnancy, (ii) taking any medication known to affect body composition or any psychoactive medication, (iii) diagnosis with a disease(s) or syndrome known to affect

body composition or fat distribution, (iv) if they had a learning impairment(s) that would complicate survey administration, (v) braces, a pacemaker, or any other contraindications to magnetic resonance imaging scanning, or (vi) participation in a weight loss, dietary, or physical intervention in the previous six months. Of the 791 eligible Hispanic students, diet recalls were conducted in 100 subjects for the original purpose of this study, and one subject was excluded due to extremely low carbohydrate intake of less than 5% of total daily intake. Seventy-eight of the remaining subjects contributed a fecal sample, but 76 were analyzed due to unreadable labels on two of the samples. Adiposity measures were taken via MRI and blood glucose and lipids were taken via fasting blood draw. Three subjects had an unquantifiable MRI, five had blood draw difficulties, and one had an unreadable glucose assay. Therefore, 73 subjects had complete adiposity measures, 71 had complete lipid measures, and 70 had complete glucose measures.

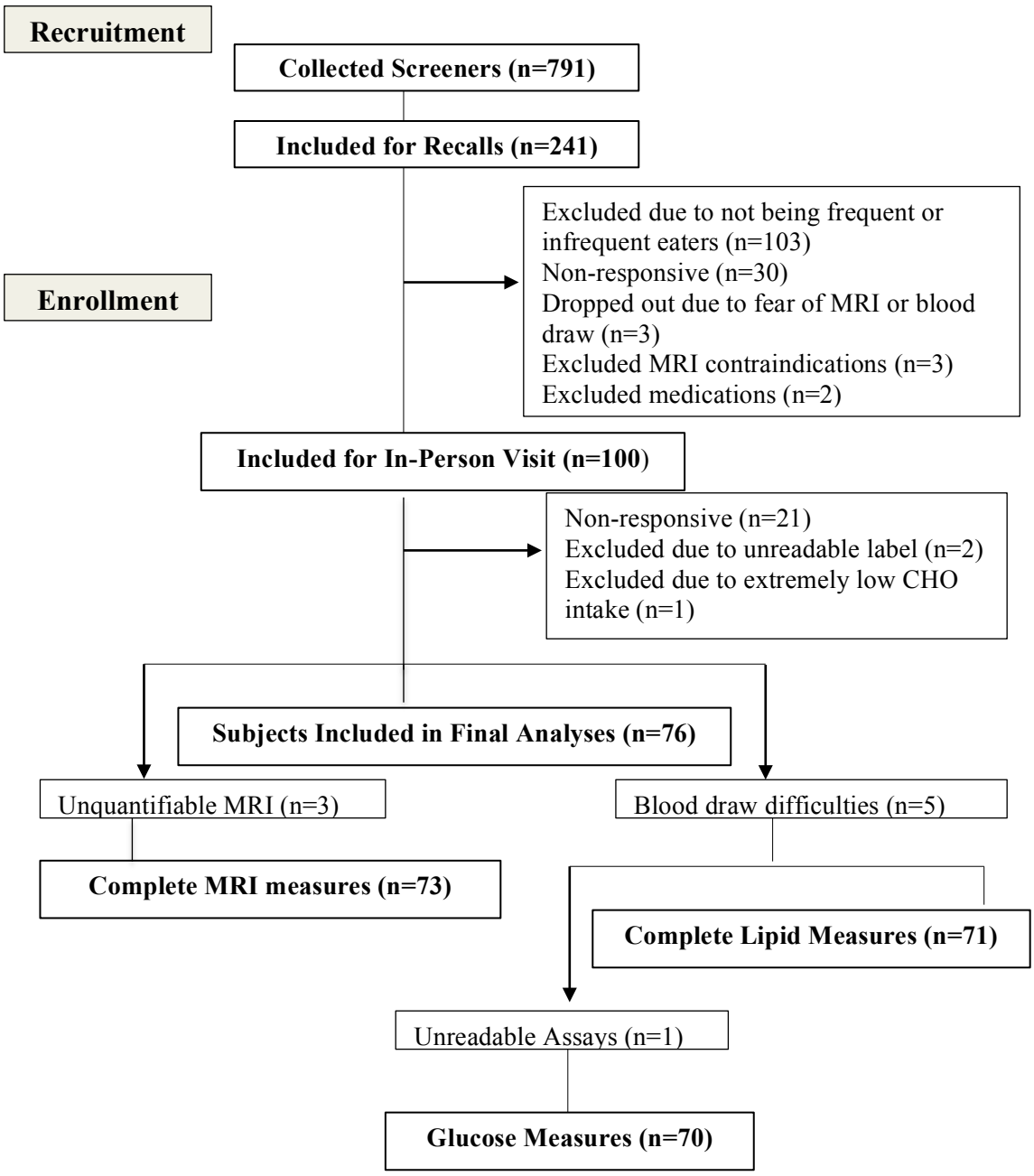

Figure 4.1: Recruitment and enrollment of study participants

Anthropometrics and Adiposity Measures

Height and weight were measured to the nearest 0.1 kg and 0.1 cm using a beam

medical scale and a wall-mounted stadiometer, respectively, and the average of two measurements was used for the analysis. BMI was calculated utilizing adult cut offs and body mass index (BMI) percentiles and z-scores (BMI-z) were determined by using EPII 2000 software (version 1.1; Centers for Disease Control and Prevention, Atlanta, GA). Subjects were categorized as overweight if they had a BMI of 25.0 to < 30.0 and obese if they had a BMI >30.0. Waist circumferences (WC) were measured and recorded to the nearest 0.1 cm. Body fat and soft lean tissue were measured using the BodPod (Cosmed 2007B, Concord, CA), which uses air displacement plethysmography. VAT, subcutaneous adipose tissue (SAT), and hepatic fat for total liver volume (HF) were assessed via MRI at the UT-Austin Imaging Research Center on a research-dedicated Siemens Skyra 3 Tesla scanner utilizing a 3D 3-point DIXON technique. An average of 26 slices were taken from the abdominal area and there was no significant difference in the number of slices between groups. The liver was then manually segmented from the volume data utilizing MATLAB (Mathwork Inc, Natick, MA).[77] Fat volume was computed on a voxel-by-voxel basis and averaged over the segmented organ. At least two research assistants quantified the fat values for each subject. No significant differences in any of the outcome variables or MRI slices were seen between research assistants utilizing t-tests. NAFLD was defined as subjects with >5.56% liver fat for total liver volume.[37–39]

Fasting Blood Draws

A fasting blood sample was obtained using a certified phlebotomist. Samples were spun to serum at the time of collection and frozen at -80 C in the freezers of Dr. Davis at the UT-Austin until the assays were performed by the Department of Medicine-Athero & Lipo in Baylor College of Medicine. Lipids were assayed using Vitros Colorimetric assays (Johnson and Johnson Clinical Diagnostics Rochester, NY) for cholesterol, triglycerides, low-density lipids (LDL), and high density lipids (HDL). Glucose was assayed on a Yellow Springs Instrument 2700 Analyzer (Yellow Springs, OH) using the glucose oxidase method. Insulin was assayed using a specific human insulin ELISA kit from (Linco, St. Charles, MO). Homeostatic model assessment (HOMA-IR) was calculated using the insulin resistance equation.[78,79]

Stool Samples

Participants were given a kit for stool sample collection with instructions and a brief questionnaire regarding past gastrointestinal illness, antibiotic use, and supplement use within one week of their in-person visit. Participants were instructed to collect the first stool of the day with a pre-moistened sample wipe, which was stored in a labeled biosafety bag and picked up by research staff within 24-hours of collection. Seventy-nine subjects returned a stool sample. Two of those subjects were excluded due to unreadable identifiers on the stool sample (n=77). Fecal DNA was stored in a -80 freezer and extracted by trained research staff using the Fecal DNA Isolation Kit (Zymo Research,

cat. no. D6010. Coded specimen was then shipped to the Microbiome Resource Laboratory (Birmingham, Alabama) to undergo 16S ribosomal RNA gene sequencing.[66]

The DNA was amplified via polymerase chain reaction (PCR) using degenerate primers flanking the V4 region of the rRNA gene to generate a 250 base pair amplicon. The individual samples were electrophoresed on agarose gel and visualed by UV illumination. The PCR product was excised and purified using a commercial (QIA) gel extraction kit (Qiagen, cat. No. 28704). The purified PCR products were then quantitated using Pico Green dsDNA reagent then sequenced using the NextGen sequencing Illumina Miseq platform from both the 5' prime and the 3' end.[66]

Microbial Composition Analysis

Fecal microbiome composition was analyzed in the microbiome analysis package Quantitative Insights Into Microbial Ecology (QIIME) v1.9.0 and simplified with QWRAP, an online statistical software tool to test differences in the microbe composition within groupings.[66] Standard methods were performed for quality control, generating abundance, descriptive statistics of sample bacteria, significant differences between groups, and the specific bacteria between the groups contributing to those differences.[64] Within QWRAP, a quality control check was done using FASTQC v0.11.2 and FASTX v0.0.13 in which all of the raw data are trimmed to reads with over at least 80% base-pairs retained.[66] Counts per sample ranged from 12,700 to 187,656. Clusters of reads with sequence similarity above a 97% cutoff were binned into Operational Taxonomic Units (OTUs), which were counted and used to measure relative abundance.[64,66] Rarefaction

curves were generated to ensure sufficient depth and measure alpha diversity.[64,66] A PERMANOVA compared the alpha diversities between tertiles with Monte Carlo permutations.[66] A p-value of < 0.05 was used to determine significance. Unweighted UniFrac, weighted UniFrac, and Bray-Curtis analysis was used to estimate beta diversity, which calculates distance as a measure of similarity of microbial communities between samples or groups of samples.[66] Kruskal-Wallis tests were used to identify differential abundant bacterial phylotypes. A p-value of <0.05 with a false discovery rate (FDR) of <0.2, as was used in the Human Microbiome Project (HMP),[19] determined significance in order to adjust for multiple hypothesis tests.[107] Microbiome composition was then examined for differences between tertiles of adiposity and metabolic measures.

Adiposity and Metabolic Groupings

Adiposity and metabolic measures were split into tertiles using SPSS version 20.0 (SPSS, Chicago, IL) and displayed in **Table 4.1**. Adiposity and metabolic measures were also grouped according to any available recommendations of healthy values for this age group and listed in **Table 4.2**.[108–111] The classifications were performed separately by two different researchers and compared for reliability.

Table 4.1: Adiposity and cardiometabolic tertile cut-offs

Adiposity & Cardiometabolic Variables	Low Range	Moderate Range	High Range
Adiposity			
VAT (ml)	< 186.7	186.7–257.7	> 257.3
SAT (ml)	< 640.9	640.9–992.1	> 992.1
WC (cm)	< 80.3	80.3–85.2	> 85.2
HF (%)	< 4.1	4.1–5.0	> 5.0
Body Fat (%)	< 22.6	22.6–30.5	> 30.5
Lipids			
HDL (mg/dL)	< 51.2	51.2–59.3	> 59.3
Cholesterol (mg/dL)	< 138.9	138.9–165.8	> 165.8
Triglyceride (mg/dL)	< 60.0	60.0–89.8	> 89.8
LDL (mg/dL)	< 74.0	74.0–89.9	> 89.9
Glucose/Insulin			
Glucose (mg/dL)	< 87.0	87.0–92.0	> 92.0
Insulin (mU/dL)	< 6.2	6.2–10.0	> 10.0
HOMA-IR	< 1.39	1.3–2.1	> 2.1

Table 4.2: Recommendations for adiposity and cardiometabolic variables

Adiposity & Cardiometabolic Variables	Healthy Range	n (%)
Adiposity Anthropometrics (n=76)[a]		
WC (cm)	M: < 88.7 / F: < 83.1	46 (61)
BMI	Normal weight	57 (75)
Body Fat (%)[b]	M: < 29.5 / F: < 41.0	66 (80)
Adiposity MRI (n=73)		
NAFLD	No disease	57 (78)
Lipids (n=71)		
HDL (mg/dL)	> 40 mg/dL	67 (96)
Cholesterol (mg/dL)	< 200 mg/dL	69 (97)
Triglyceride (mg/dL)	< 150 mg/dL	68 (96)
LDL (mg/dL)	< 130 mg/dL	71 (100)
Glucose/Insulin (n=70)		
Glucose (mg/dL)	< 100 mg/dL	65 (93)
Insulin (mU/mL)	< 25 mU/mL	68 (97)
HOMA-IR: American	< 2.6	58 (83)
HOMA-IR: Mexican-American	< 3.8	68 (94)

[a] No recommendations for VAT, SAT [b] Recommendations specific to Mexican-Americans aged 16-19

RESULTS

Demographics and cardiometabolic measures are displayed in **Table 4.3**. The average age of participants was 18 years old, 45% were male, 25% were overweight or obese, and 22% had NAFLD. Averages of all the other adiposity and cardiometabolic variables were within the acceptable ranges for this age group.[108,111–113]

Table 4.3: Recommendations for adiposity and cardiometabolic variables. Data presented in mean ±SD or n (%)

Subject Characteristics (n=76)	
Sex M	34 (44.5%)
Age (y)	18.7 ±0.4
Overweight or Obese Prevalence	19 (25%)
Body Fat (%)	26.6 ±10.0
Waist Circumference (cm)	84.4 ±9.7
Adiposity (n=73)	
Visceral Adipose Tissue (ml)	253.4 ±128.2
Subcutaneous Adipose Tissue (ml)	973.37 ±637.6
Hepatic Fat Fraction (%)	5.9 ±5.5
Prevalence of NAFLD (%)	16 (22%)
Lipids (n=71)	
HDL (mg/dL)	55.0 ±11.3
Cholesterol (mg/dL)	153.0 ±22.4
Triglyceride (mg/dL)	80.7 ±33.7
LDL (mg/dL)	81.7 ±19.1
Glucose/Insulin (n=70)	
Glucose (mg/dL)	89.4 ±7.8
Insulin (mU/mL)	8.6 ±4.9
HOMA-IR	1.1 ±1.3

Body fat, LDL, and insulin had significant beta-diversity between groups (unweighted 0.02, 0.02, and 0.04, respectively). The mean values of significant tertiles were as follows: body fat– low: 15.7 ±5.0%, moderate: 26.2 ±2.4%, and high: 38.0 ±4.8%; LDL– low: 60.7 ±9.2 mg/dL, moderate: 81.7 ±4.5 mg/dL, and high: 102.7 ±9.7 mg/dL; insulin– low: 4.3 ±1.3 mU/dL, moderate: 7.9 ±1.2 mU/dL, and high: 13.6 ±5.4 mU/dL. Tertiles of VAT, SAT, WC, HF, HDL, cholesterol, triglyceride, glucose, and HOMA-IR did not achieve significance. Bray-Curtis and weighted Unifrac p-values did

not achieve significance.

Figure 4.2 displays the significant difference in alpha-diversity in the gut microbiome between tertiles of percent body fat, insulin, and LDL. Subjects with high body fat percent compared to those with low and moderate body fat percentages had lower alpha-diversity levels (4.8 ±0.8 vs. 5.1 ±0.7 and 5.2 ±0.5; p=0.02). Subjects with high and moderate insulin levels compared to those with low insulin values had lower alpha diversity levels (4.9 ±0.9 and 4.9 ±0.6 vs. 5.3 ±0.6; p=0.02). Subjects with high LDL levels compared to subjects with low and moderate LDL had increased diversity (5.3 ±0.6 vs. 4.9 ±0.5 and 5.0 ±0.9; p=0.04).

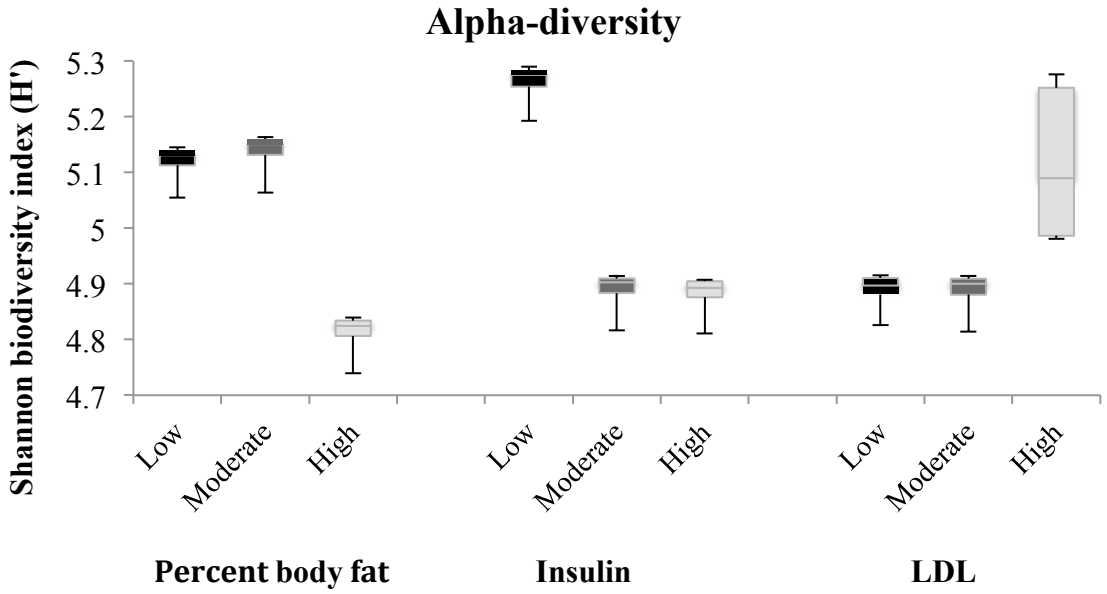

Figure 4.2: Microbiome alpha-diversity between subjects. Subjects with low percent body fat and low levels of insulin had a more diverse microbiome compared to subjects with high percent body fat and high insulin levels (p=0.02 and p=0.04, respectively). Subjects with high levels of LDL had a more diverse microbiome compared to subjects with low LDL levels (p=0.02).

Participants classified as either healthy or unhealthy using the recommendation parameters and the majority of subjects achieved the healthy recommendations. None of the variables achieved significance including NAFLD and BMI.

Table 4.4 displays the bacteria by taxonomic classification that are significantly different between tertiles of percent body fat. Bacteria at the species level did not achieve significance. Participants with high percent body fat compared to participants with low and moderate body fat percentages had significantly less relative abundance of *Methanobrevibacter*, *Akkermansia,* and *Clostridiales* and significantly more relative abundance of *Fusobacteriales* and *Bacteroidia*.

Table 4.4: Differences in bacteria between tertiles of body fat.

Taxonomic Classification	Bacteria	Mean relative abundance (%)			*p* value	*FDR*
		Low	Moderate	High		
Phylum	*Euryarchaeota*	0.00036	0.00038	0.00005	0.00	0.02
Phylum	*Verrucomicrobia*	0.01035	0.00268	0.00057	0.00	0.02
Phylum	*Fusobacteria*	0.00649	0.00131	0.00420	0.02	0.09
Phylum	*Bacteroidetes*	0.00090	0.00112	0.00084	0.04	0.09
Class	*Methanobacteria*	0.00036	0.00038	0.00005	0.00	0.03
Class	*Verrumicrobiae*	0.01010	0.00247	0.00056	0.00	0.03
Class	*Fusobacteriia*	0.00064	0.00131	0.00420	0.04	0.16
Class	*Bacteroidia*	0.30292	0.31076	0.38727	0.04	0.16
Class	*Clostridia*	0.58170	0.57095	0.47327	0.02	0.15
Order	*Methanobacteriales*	0.00036	0.00038	0.00005	0.00	0.04
Order	*Verrucomicrobiales*	0.01011	0.00247	0.00056	0.00	0.04
Order	*Fusobacteriales*	0.00064	0.00131	0.00420	0.02	0.19
Order	*Clostridiales*	0.58170	0.57095	0.47327	0.02	0.19
Family	*Methanobateriaceae*	0.00036	0.00038	0.00005	0.00	0.09
Family	*Verrucomicrobiaceae*	0.01010	0.00247	0.00056	0.00	0.09
Genus	*Methanobrevibacter*	0.00036	0.00038	0.00005	0.00	0.19
Genus	*Akkermansia*	0.01010	0.00247	0.00005	0.00	0.19

Methanobrevibacter belongs to family *Methanobateriaceae*, order *Methanobacteriales*, class *Methanobacteria*, and phylum *Euryarcheota*; *Akkermansia* belongs to family *Verrucomicrobiaceae*, order *Verrucomicrobiales*, class *Verrucomicrobiae,* and phylum *Verrucomicrobia*; and *Clostridiales* belongs to class *Clostridia*; *Fusobacteriales* belongs to class *Fusobacteriia* and phylum *Fusobacteria*; and *Bacteroidia* belongs to the phylum *Bacteroidetes;* all of which achieved significance.

Participants with high and low levels of insulin compared to participants with moderate levels of insulin had significantly less relative abundance of phylum *Proteobacteria* as displayed in **Table 4.5**.

Table 4.5: Differences in bacteria between tertiles of insulin.

Taxonomic Classification	Bacteria	Mean relative abundance (%)			*p* value	*FDR*
		Low	Moderate	High		
Phylum	*Proteobacteria*	0.04171	0.21222	0.03874	0.02	0.15

Table 4.6 lists the bacteria by taxonomic classification that are significantly different between tertiles of LDL. Participants with high levels of LDL compared to participants with low and moderate levels of LDL had significantly higher relative abundance of *anthropi, europaeus, Pyramidobacter*, *Mobilincus, Campylobacter*, *Facklamia, Gallicola*, *WAL_1855D*, *1-68*, *Mogibacterium*, and *Peptococcus*.

Table 4.6: Differences in bacteria between tertiles of LDL.

Taxonomic Classification	Bacteria	Mean relative abundance (%)			*p* value	*FDR*
		Low	Moderate	High		
Phylum	*Synergistetes*	0.00014	0.00000	0.00126	0.00	0.02
Class	*Synergistia*	0.00014	0.00000	0.00126	0.00	0.04
Class	*Epsilonproteobacteria*	0.00243	0.00184	0.01016	0.01	0.19
Order	*Synergistales*	0.00014	0.00000	0.00126	0.00	0.03
Family	*Dethiosulfovibrionaceae*	0.00014	0.00000	0.00126	0.00	0.07
Family	*Aerococcaceae*	0.00014	0.00118	0.00137	0.00	0.07
Genus	*Jonquetella*	0.00000	0.00000	0.00082	0.00	0.18
Genus	*Pyramidobacter*	0.00014	0.00000	0.00043	0.01	0.18
Genus	*Campylobacter*	0.00243	0.00184	0.01015	0.01	0.18
Genus	*Facklamia*	0.00013	0.00114	0.00111	0.01	0.18
Genus	*Mobiluncus*	0.00049	0.00074	0.00457	0.00	0.08
Genus	*Gallicola*	0.00051	0.00021	0.00141	0.01	0.18
Genus	*WAL_1855D*	0.01474	0.01084	0.03263	0.01	0.18
Genus	*1-68*	0.00500	0.00403	0.00786	0.01	0.18
Genus	*Moryella*	0.00009	0.00000	0.00048	0.01	0.18
Genus	*Mogibacterium*	0.00112	0.00056	0.00140	0.02	0.18
Genus	*Peptococcus*	0.00060	0.00035	0.00161	0.02	0.19
Species	*anthropi*	0.00000	0.00000	0.00082	0.00	0.19
Species	*europaeus*	0.00003	0.00002	0.00321	0.00	0.14

Species *anthropic* belongs to genus *Jonquetella*, family *Dethiosulfovibrionaceae*, order *Synergistales,* class *Synergistia,* and phylum *Synergistetes*. Genus *Pyramidobacter* belongs to the same taxonomic classifications. Genus *Campylobacter* belongs to class *Epsilonproteobacteria*. Genus *Facklamia* belongs to family *Aerococcaeceae*. All of the aforementioned achieved significance.

DISCUSSION

This is the first study to examine the relationship between cardiometabolic measures and the microbiome in an exclusively Hispanic college population.

Approximately one quarter of this population was overweight or obese and had NAFLD. Percent body fat, insulin, and LDL were the main variables linked to an altered microbiome. Subjects with high percent body fat compared to those with low and moderate percent body fat had lower microbial composition diversity, confirming several studies in which obesity was associated with a significant decreased level of diversity.[24,30,75,114] Subjects with high insulin levels compared to subjects with moderate and low insulin levels also had lower microbial composition diversity. This was expected since dysbiosis and decreases in microbial biodiversity have been associated with metabolic disease states, including diabetes.[22,31,70]

Contrary to our hypothesis, participants with low and moderate LDL levels compared to subjects who had high values had lower diversity. However, upon further analysis, the range of the LDL was large and the mean of the high LDL group was near optimal range with 100% of subjects meeting the recommendations for LDL. Some research suggests that low levels of LDL are correlated with adverse health effects.[115–117] Since LDL is a carrier protein for cholesterol, which is a critical component of cell membranes and sex hormones, subjects closer to the optimal range of LDL would have an increased microbial diversity. A more unhealthy population is needed to study the impact beyond low and optimal levels of LDL.

An increase of percent body fat was associated with lower relative abundance of *Methanobrevibacter, Akkermansia*, and *Clostridiales*. These bacteria have been classified as components of a healthy gut. In recent human studies, *Methanobrevibacter* has been associated with leanness, and is found to be prevalent in the healthy human colon

compromising up to 10% of the microbiome.[118] *Akkermansia* is a mucin-degrading bacteria that represents 3-5% of the microbial community in healthy subjects and is inversely correlated with body weight.[114,119,120] In addition, probiotic administration of *Akkermansia* was found to restore abundance and improve gut barrier and metabolic parameters in diet-induced obese mice.[119] *Clostridiales* has important roles in the metabolism of dietary fiber and was identified as the most active microbial component in healthy adult intestinal environments.[121–123] The current study confirms these findings and suggests that certain gut bacteria act as protective barriers against obesity.

Moderate values of insulin were associated with a decreased relative abundance of *Proteobacteria* compared to both high and low value groups. However, *Proteobacteria* is a phyla that encompasses bacteria of extreme metabolic diversity.[124] Therefore, no conclusions about *Proteobacteria* and insulin can be made without further specificity. This inconclusive data is most likely due to the participants meeting the recommended range of insulin, and therefore may all have similar microbes that aid in insulin management.

Low values of LDL were associated with decreased relative abundance of *Jonquetella anthropi, Actinomyces europaeus, Pyramidobacter, Peptococcus, Moryella, Mobilincus,Campylobacter, Facklamia, Mogibacterium, Gallicola, WAL_1855D,* and *I-68*. Some of these bacteria have designated functions, while the functions of others have not yet been found or are not clear. *Jonquetella anthropic, Actinomyces europaeus*, and *Pyramidobacter* aid in producing short chain fatty acids which are metabolically beneficial by decreasing inflammation.[61,125–128] *Peptococcus* are involved in circulation of

steroid molecules from the liver, which is the function of LDL and attests to a possible mechanism. *Moryella* ferment carbohydrates to produce indoles, which have diverse biological roles such as intercellular signaling.[129,130] *Mobilincus, Campylobacteria, Facklamia, Mogibacterium, Gallicola, WAL_1855D,* and *I-68* have unclear functions. It is important to note, that some of these bacteria have been associated with infections such as diarrhea or bacterial vaginosis, but their role or proof of pathogen has not been proven and is speculative.[131–134] These results suggest that having too low values of LDL may negatively affect the microbiome or vice versa. Having ranges near the optimal cut off is correlated with having non-inflammatory metabolic products. However, a wider range of LDL values are needed to draw conclusions between healthy and unhealthy subjects. In addition, the specific functions of bacteria in humans remain elusive and further examination is needed.

There were no significant differences in the gut microbiome between tertiles of VAT, SAT, WC, and HF. Participants who were overweight or obese compared to normal weight participants did not have differences in the gut microbiome. Of note, this population was healthier than most. Only 25% were classified as overweight or obese, which is significantly less than the national average for Hispanics in this age range of 38.9%.[2] In addition, over 96% of subjects met the recommend ranges for lipids and over 83% for insulin resistance. None of the subjects had metabolic syndrome or diabetes. Percent body fat did achieve significance and is a more comprehensive and direct measure for body fat.

There were no differences in gut microbiome between participants with NAFLD

and those without. NAFLD has been associated with an altered gut microbiome.[67] Although, like the current study, several human studies confirm there is no significant difference in overall diversity of microbial composition diversity between participants with NAFLD and their controls.[42,43] When further examined, the only significant bacteria in this population was the order *Verrucomicrobiales* and subjects with NAFLD had lower amounts. This bacterium is part of the phylum *Verrucomicrobia,* which only has two species detected in the human gastrointentstinal tract. A decreased abundance in those species are linked to compromised health.[134] However, this bacteria was not significant in other studies and participants with NAFLD have shown different abundances of bacteria for different ages and ethnic groups.[40,43]

There were no significant differences in cholesterol, triglyceride, HDL, glucose, and insulin resistance. An animal study showed the gut microbiome was a moderating link between dietary choline and the progression of atherosclerosis and increased cholesterol[105]. So although HDL was approaching significance, these null results are most likely due to this population being within recommended ranges for these measures.

The limitations of this study include its cross-sectional design and small sample size. This population was homogenous in ethnicity, location, and age, eliminating possible confounders which could be a strength but also a possible weakness. In addition, this population being metabolically healthy did not provide a contrast to unhealthy groups. A larger sample size, more diversity in metabolic values, and deeper amplification are needed to further explore significant differences in specifying bacteria available in smaller amounts. Also, the specific functions of bacteria need further

exploration.

In summary, few studies have implicated human cardiometabolic measures on the decreased diversity of the microbiome. Metabolic diseases disproportionally affect Hispanics and no studies have examined adiposity and metabolic measures on the microbiome in an exclusively Hispanic college population. [11] These findings suggest that participants with lower percentages of total body fat, lower values of insulin, and optimal values of LDL contain a higher relative abundance of a multitude of beneficial bacteria than those with more total body fat and insulin and low values of LDL. These bacteria suggest a possible mechanism for protection against cardiovascular and metabolic disease. More longitudinal and intervention studies are warranted to understand the role that diet plays on the gut microbiome and subsequent disease risk.

Chapter 5: Saturated fat intake correlates with altered composition of fecal microbiome in Hispanic college students

Pilles KN, Van Der Pol WJ, Morrow CD, Asigbee FM, Bray MS, Davis JN.

ABSTRACT

Background: The college years is a critical transition period in which lifetime eating habits are established and Hispanics are disproportionately affected by obesity and related metabolic disease. Significant and meaningful changes in the gut microbiota have been associated with dietary alterations, primarily consumption of dietary fiber, fat, sugar, and being breastfed during infancy, but the mechanism and magnitude of influence is unknown. In addition, the microbiome of an exclusive Hispanic population as well as college-aged students have not been studied. Therefore, understanding how diet impacts the composition of the fecal microbe community in Hispanic college students is warranted.

Objective: The purpose of this study is to examine the relationship between diet and the gut microbiome in Hispanic college freshmen.

Design: Dietary intake via multiple 24-hour dietary recalls and stool samples were collected from 76 Hispanic college freshmen (18-19 y). Dietary variables were grouped according to current recommendations and subjects were classified as either met recommendations or exceeded recommendations.

Results: Sixty-two percent of subjects exceeded the recommendations for saturated fat. Significant differences in microbial diversity were found between those who met recommendations and those who exceeded recommendations for saturated fat as

determined from unweighted Uni-Frac analysis. Those who exceeded saturated fat recommendations compared to those who met them had significantly less relative abundance of *Methanobacteriales, Victivallales, Bacillales, and Campylobacteria*.

Conclusion: Few human studies have collected extensive dietary data and microbiome diversity, and this is the first study to examine the relationship between dietary intake and the microbiome in an exclusively Hispanic college population. Our findings support previous findings in animal models that diets high in saturated fat correlated with decreased diversity in the microbial composition reflected by increased abundance of *Firmicutes*. This study suggests that a decrease in saturated fat intake is recommended in order to increase biodiversity and thereby decrease inflammatory diseases.

INTRODUCTION

College is known to be a transitional period of time when young adults in the United States (US) consume more junk food and alcohol, and less dietary fiber, fruits and vegetables.[4–6] Several studies have shown that 70% of college freshmen gain an average of 3.5 to 7.7 pounds in the first year of college[4,7–9] with no difference in dietary intake and physical activity from their freshman year to their senior year.[4,10] Therefore, the transition to college has been identified as a critical period contributing to the rise in obesity rates as the behavioral choices college students make likely affect their risk of chronic disease later in life.

Hispanics are the largest and fastest growing ethnic minority in the US and in recent history have surpassed Non-Hispanic Whites and Blacks in college enrollment.[1,2]

In 2016, Hispanic students represented 23% of freshman enrollment at the University of Texas at Austin (UT-Austin), having the largest increase among all minority groups.[106] Hispanics are also disproportionately affected by obesity, type-2 diabetes (T2D), and non-alcoholic fatty liver disease.[11] Diets high in added sugar and low in dietary fiber as well as decreased eating frequency and skipping breakfast have been positively linked to obesity levels, visceral adipose tissue, insulin resistance, and circulating lipids in Hispanic youth and young adults.[12–17]

The human gut is a host to a diverse and dynamic community of microbes that encode proteins not found in the human genome and that play numerous diverse roles in metabolism and energy homeostasis. Current research suggests that disruptions in the normal balance of gut microbial populations are linked to a variety of gut-related disease and conditions, such as metabolic syndrome and obesity.[18,22,23] Although what constitutes a "healthy" gut microbiome remains unknown, it is clear that diversity and abundance of microbial populations is essential. A decrease in microbial diversity have been demonstrated in multiple disease states, including obesity, inflammatory bowel disease, T2D, and colorectal cancer.[18,25,26,61]

Significant and meaningful changes in the gut microbiota have been associated with dietary alterations, primarily consumption of dietary fiber, fat, sugar, and being breastfed during infancy.[25–27,61] However, the mechanism and magnitude of dietary intake influence on the composition of the gut microbiome is not clear. No study has examined the relationship between the microbiome and diet exclusively in a high-risk Hispanic young adult (ages 18-19) population. In addition, although breakfast composition has

been explored[73,135–137] no study has examined the effect of breakfast intake versus skipping breakfast, nor examined the influence of eating frequency on the gut microbiome. Given that Hispanics are disproportionately affected by obesity and metabolic disease and college years are a critical transition period in which lifetime eating habits are established, understanding how dietary intake impacts the gut microbiome in this population is warranted. Thus, the **overall goal** of this study is to examine the relationship between diet and the gut microbiome in Hispanic college freshmen.

SUBJECTS AND METHODS

Participants

Figure 5.1 provides a detailed flow of study participants. The original purpose of this study was to examine the relationship between eating frequency and adiposity and metabolic markers. Hispanic college freshmen subjects were recruited via announcement in classes, word of mouth, electronic posted notices, and tabling at dorms around the UT-Austin campus. Subjects completed a screener to determine eligibility. Inclusion criteria included: (i) self-report that all four of their grandparents were of Hispanic origin (ii) 18-19 years of age, and (iii) in their first year of college. Exclusion criteria included (i) current pregnancy, (ii) taking any medication known to affect body composition or any psychoactive medication, (iii) diagnosis with a disease(s) or syndrome known to affect body composition or fat distribution, (iv) if they had a learning impairment(s) that would complicate survey administration, (v) braces, a pacemaker, or any other contraindications to magnetic resonance imaging scanning, or (vi) participation in a weight loss, dietary, or

physical intervention in the previous six months. Of the 791 eligible Hispanic students, dietary recalls were conducted in 100 subjects. Seventy-nine of those subjects contributed a fecal sample, but 76 were analyzed due to unreadable labels on two of the samples and one subject having an extremely low carbohydrate intake of less than 5% of total daily intake.

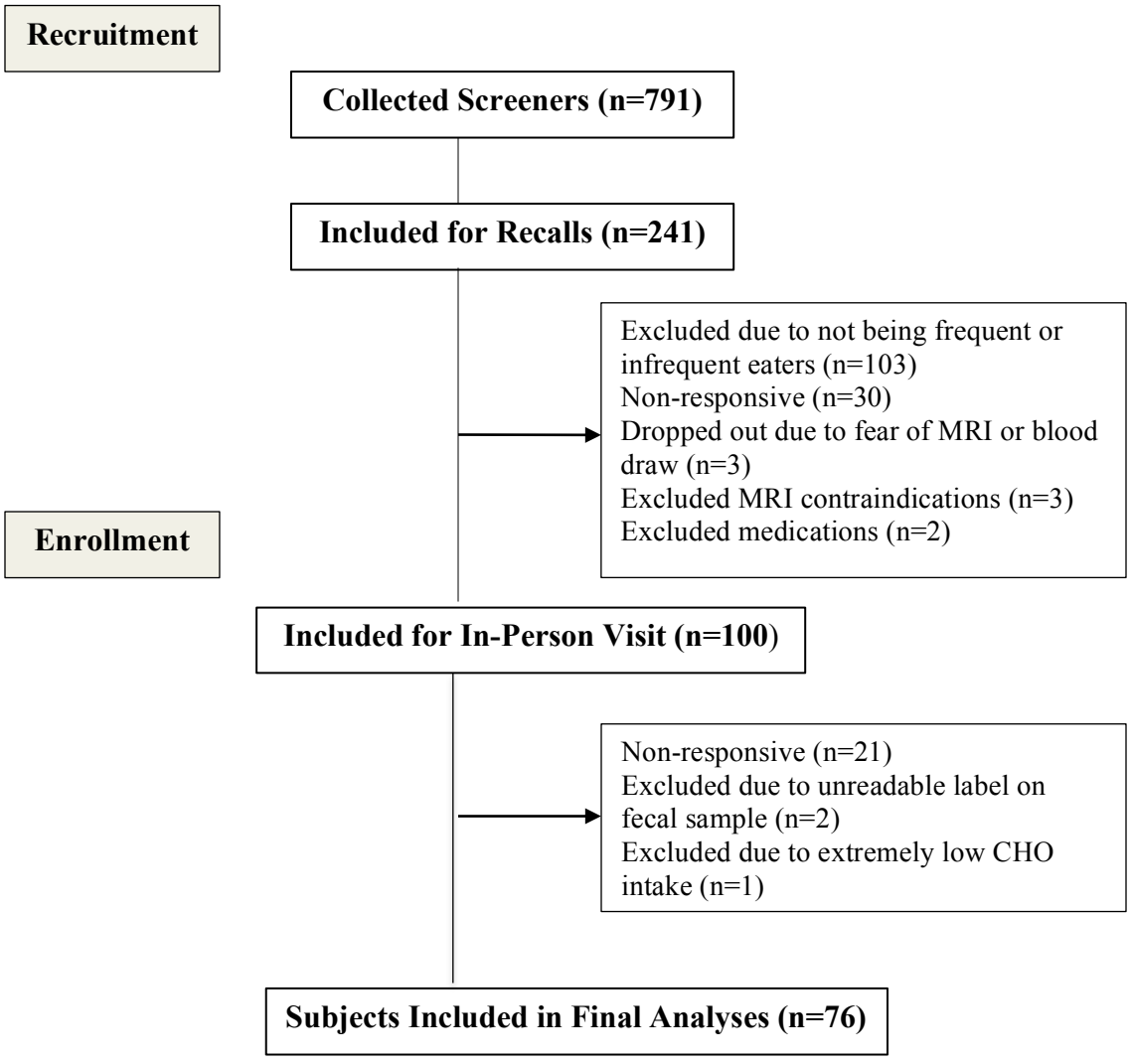

Figure 5.1: Recruitment and enrollment of study participants

Dietary Intake

Dietary intake was assessed from at least three 24-h diet recalls (one weekend and two weekdays) using the multiple-pass technique. Research staff collecting the diet recalls were trained and supervised by a Registered Dietitian. On average, recalls were administered within five days from the in-person testing visit. All dietary recall data were double-entered by trained research staff. Nutritional data was analyzed by using the Nutrition Data System for Research (NDS-R, 2014). The NDS-R program was used to calculate key dietary variables for this analysis, including mean energy intake, total fat, protein, carbohydrates, saturated fat, total sugar, added sugar, dietary fiber, soluble fiber, and insoluble fiber. Prospectively, no recall was performed if the subject indicated being ill. Plausibility of energy intake was assessed by regressing caloric intake against body mass index, and no subjects were over two standard deviations from the mean (n=99).

Stool Samples

Participants were given a kit for stool sample collection with instructions and a brief questionnaire regarding past gastrointestinal illness, antiobiotic use, and supplement use within one week of their in-person visit. Participants were instructed to collect the first stool of the day with a pre-moistened sample wipe, which was stored in a labeled biosafety bag and picked up by research staff within 24-hours of collection. Seventy-nine subjects returned a stool sample. Two of those subjects were excluded due to unreadable identifiers on the stool sample (n=77). Fecal DNA was stored in a -80 freezer and extracted by trained research staff using the Fecal DNA Isolation Kit (Zymo Research,

cat. no. D6010. Coded specimen was then shipped to the Microbiome Resource Laboratory to undergo 16S ribosomal RNA gene sequencing.[66]

The DNA was amplified via polymerase chain reaction (PCR) using degenerate primers flanking the V4 region of the rRNA gene to generate a 250 base pair amplicon. The individual samples were electrophoresed on agarose gel and visualed by UV illumination. The PCR product was excised and purified using a commercial (QIA) gel extraction kit (Qiagen, cat. No. 28704). The purified PCR products were then quantitated using Pico Green dsDNA reagent then sequenced using the NextGen sequencing Illumina Miseq platform from both the 5' prime and the 3' end. [66]

Microbial Composition Analysis

Fecal microbiome composition was analyzed in the microbiome analysis package Quantitative Insights Into Microbial Ecology (QIIME) v1.9.0 and simplified with QWRAP, an online statistical software tool to test differences in the microbe composition within groups.[66] Standard methods for quality control, generating abundance, descriptive statistics of sample bacteria, significant differences between groups, and the specific bacteria between the groups contributing to those differences.[64] Within QWRAP, a quality control check was done using FASTQC v0.11.2 and FASTX v0.0.13 in which all of the raw data are trimmed to reads with over at least 80% base-pairs retained.[66] Counts per sample ranged from 12,700 to 187,656. Clusters of reads with sequence similarity above a 97% cutoff were binned into Operational Taxonomic Units (OTUs), which were counted and used to measure relative abundance.[64,66] Rarefaction curves were generated

to ensure sufficient depth and measure alpha diversity.[64,66] Nonparametric two-sample t-tests compared the alpha diversities between two groups with Monte Carlo permutations.[66] A p-value of < 0.05 was used to determine significance. Unweighted UniFrac, weighted UniFrac, and Bray-Curtis analysis was used to estimate beta diversity, which calculates distance as a measure of similarity of microbial communities between samples or groups of samples.[66] Kruskal-Wallis tests were used to identify differential abundant bacterial phylotypes. A p-value of <0.05 with a false discovery rate (FDR) of <0.2, as was used in the Human Microbiome Project (HMP),[19] determined significance in order to adjust for multiple hypothesis tests.[107] Microbiome composition was then examined for differences between diet groups.

Dietary Groupings

Diet variables were also grouped into tertiles using SPSS version 20.0 (SPSS, Chicago, IL). Diet variables were also grouped according to current dietary recommendations.[83] Values for each participant were classified as "Met" or "Exceeded" dietary recommendations. Participants were classified as meeting recommendations for macronutrient and sugar categories if 45-65% of total daily kilocalories were composed of carbohydrates, 25-35% of total daily kilocalories were composed of fats, 10-30% of total daily kilocalories for protein, or <15% of total daily kilocalories were composed of added sugar.[83,95] For saturated fat, participants were classified as meeting recommendations if <10% of total daily kilocalories were composed of saturated fat. For dietary fiber, men with ≥38 grams of fiber/day or females with ≥25 grams of fiber/day

were classified as meeting recommendations.[83,97] If a participant fell outside of the guidelines, he/she was classified as below or above recommendations. Breakfast eaters waere defined as participants who consumed foods that constituted ≥15% of total daily energy within three hours of waking.[138] Those who ate breakfast on all three days of dietary assessments were considered ALWAYS breakfast consumers, while those who consumed breakfast on one or two days of the three days were defined as INTERMITTENT breakfast consumers, and those who never consumed breakfast were defined as NEVER breakfast consumers. Eating occasions (EO) were defined as ≥50 kilocalories and ≥15 minutes from any previous EO.[139] Participants who had on average four or more EO per day were classified as FREQUENT eaters and those who consumed on average less than three EO per day were classified as INFREQUENT eaters. The classifications were performed separately by two different researchers and compared for reliability.

RESULTS

Participant Characteristics

Demographics and dietary intake are displayed in **Table 5.1**. The average age of participants was 18 years old, 55% were female, and 25% were overweight or obese. Fifty percent of participants exceeded recommendations for total fat and 62% exceeded recommendations for saturated fat. Ninety-seven percent of participants met protein recommendations and 66% met carbohydrate recommendations. Twenty-five percent met added sugar recommendations, but most consumed less than the recommended limit,

whereas only 5% met fiber recommendations.

Table 5.1: Demographics and diet of study participants. Data presented in mean ±SD or n (%).

Subject Characteristics (n=76)	
Sex M/F	34/42
Age (y)	18.7 ±0.4
BMI	23.6 ±3.7
Overweight or Obese	19 (25)
Dietary Variables (n=76)	
Energy intake (kcal/d)	1973.6 ±732.7
Fat (% daily kcal)	34.3 ±5.6
Saturated Fat (% daily kcal)	10.8 ±2.5
Protein (% daily kcal)	17.7 ±4.7
Carbohydrate (% daily kcal)	47.8 ±7.5
Added Sugar (% daily kcal)	3.3 ±1.8
Total Fiber (g)	17.2 ±7.5

Characterizing the Hispanic gut microbiome

Figure 5.2 shows Hispanic freshmen college students have microbiomes primarily composed of 57.2% *Firmicutes*, 33.2% *Bacteroidetes*, 5.3% *Actinobacteria*, 3.3% *Proteobacteria*, 0.5% *Verrumicrobia*, and 0.01% *Other*. Compared to HMP, a healthy population of Americans, and MetaHIT, a healthy population of Europeans,[69] FHS had percentages of bacteria that fall within the range of the two healthy populations with the exception of *Actinobacteria* which had more relative abundance than HMP and MetaHIT (5% vs. 0% and 2%, respectively).

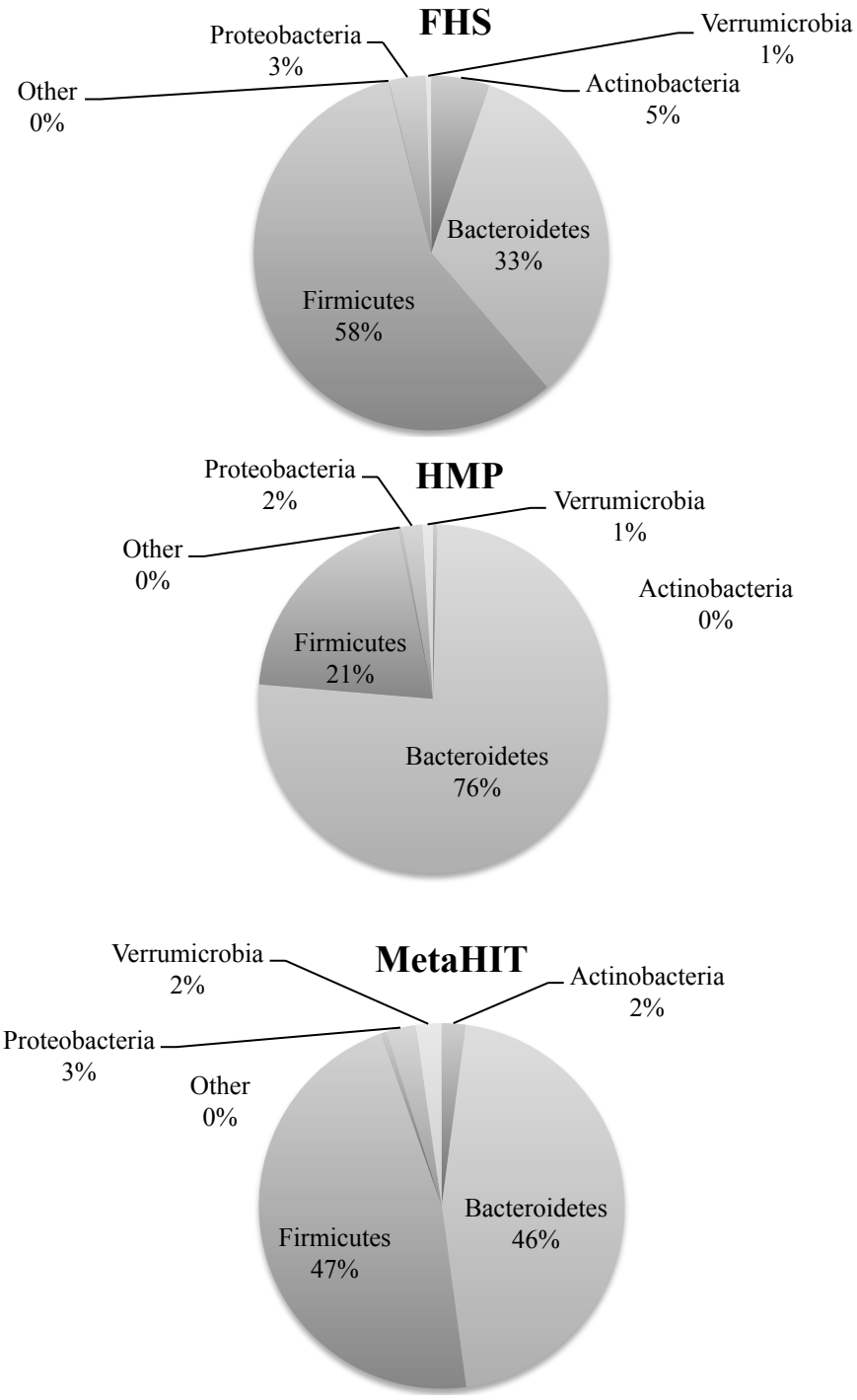

Figure 5.2: Quantitative comparison of relative taxonomic abundance of fecal microbiota in the Freshmen Health Study compared to HMP and MetaHIT.

Comparing beta-diversity by tertiles and by recommendations, saturated fat was the only dietary variable with significant differences, thus the data is only presented using saturated fat recommendations (i.e., those who met vs. those who exceeded recommendations). Compared to subjects who met dietary recommendations to those who did not, saturated fat was the only dietary variable with significant beta-diversity differences (un-weighted p=0.01) as seen in **Table 5.2**. Bray-Curtis and weighted p-values did not achieve significance. The gut microbiome was not significantly different in other dietary variables, such as subjects who met recommendations for carbohydrates, total fat, protein, total fiber, insoluble fiber, soluble fiber, total sugar, added sugar. In addition, breakfast intake and eating frequency were not significantly linked to the gut microbiome. When dietary variables were grouped according to tertiles, saturated fat was again the only dietary variable contributing a significant difference (p=0.00) in microbiome composition (data not shown). Tertiles of carbohydrates, total fat, protein, total fiber, insoluble fiber, soluble fiber, total sugar, added sugar were not significantly linked to the gut microbiome. **Figure 5.3** shows the Shannon biodiversity index of increased diversity of the microbiome in subjects who met saturated fat recommendations compared to those who exceeded recommendations (5.21 ±0.90 vs. 4.92 ±0.52; p=0.01).

Table 5.2: Beta-diversity between groups of recommendations of dietary variables. Category data presented in n (%); p-value is unweighted

Dietary Nutrients	**Categories**			**β-diversity p-value**
	Below	*Met*	*Exceeded*	
Carbohydrates	26 (34)	50 (66)	0 (0)	0.43
Fat	3 (4)	38 (50)	35 (46)	0.78
Protein	0 (0)	74 (97)	2 (3)	0.60
Added Sugar	64 (84)	12 (16)	0 (0)	0.77
Saturated Fat	0 (0)	29 (38)	47 (62)	0.01*
Total Fiber	72 (95)	4 (5)	0 (0)	0.12
Dietary Patterns	**Categories**			**β-diversity p-value**
Breakfast Consumption	*Never*	*Intermittent*	*Always*	
	17 (22)	45 (59)	14 (18)	0.66
Eating Frequency	*Infrequent*		*Frequent*	
	34 (45)		42 (55)	0.52

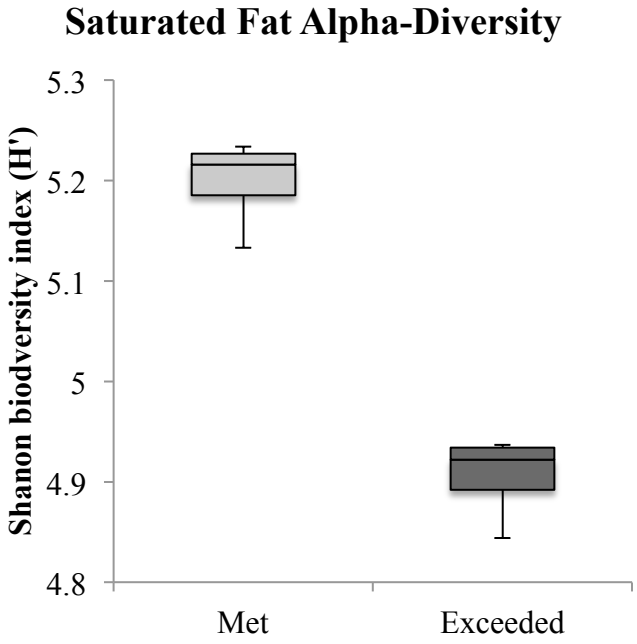

Figure 5.3: Microbiome biodiversity between subjects who met and exceeded saturated fat recommendations. Those who exceeded saturated fat recommendations compared to those who met them had less diversity in the human gut microbiome (overall biodiversity 4.92 ±0.52 vs. 5.21 ±0.90; p=0.01)

Participants who exceeded saturated fat recommendations compared to participants who met saturated fat recommendations had significantly less relative abundance of order *Victivallales* (1.4 x 10^4 vs. 1.0 x 10^5; p=0.04, FDR 0.18) member of the *Lentisphaeria* class and *Lentisphaerae* phylum, order *Methanobacteriales* (5.3 x 10^4 vs. 9.0 x 10^5p=0.03, FDR 18%) member of the *Methanobacteria* class and *Euryarchaeota* phylum, order *Campylobacterales* (7.5 x 10^3 vs. 2.7 x 10^3; p=0.04, FDR 18%) member of the *Epsiloprotebacteria* class, and order *Bacillales* (3.8 x 10^3 vs. 1.2 x 10^3; p=0.04, FDR 18%) while linked to inverse effects in order *Other* as seen in **Figure 5.4.** Genus and

species did not achieve significance in any of the bacteria.

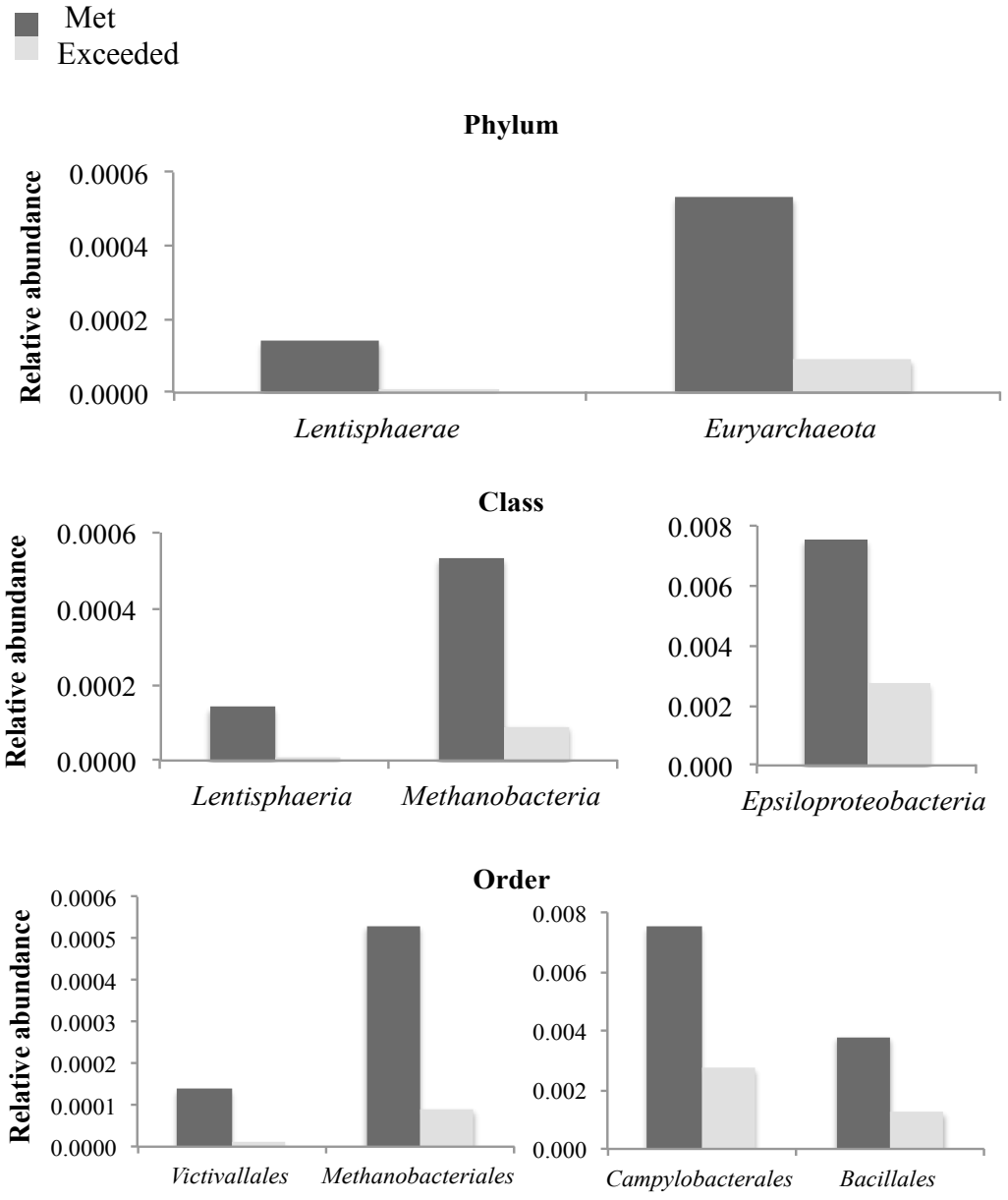

Figure 5.4: Relative abundance of significant bacteria between participants who met and exceeded saturated fat recommendations. Data presented in mean relative abundance, with $p < 0.05$ and FDR < 0.2.

DISCUSSION

This is the first study to examine the relationship between dietary intake and the microbiome in an exclusively Hispanic college population. *Firmicutes* were the dominant bacteria found in this population. Saturated fat was the main dietary variable contributing to an altered microbiome. Those who exceeded saturated fat recommendations compared to those who met it had lower microbial composition diversity, reflected by a decreased relative abundance of *Victivallales, Methanobacteriales, Campylobacterales*, and *Bacillales*.

Decreases in microbial biodiversity are linked to metabolic disease states and a more diverse microbiome is linked to a healthy profile.[140] In a study comparing mice fed a normal chow diet to mice fed a high fat diet, mice consuming a high fat diet had significantly lower diversity than their normal chow fed counterparts.[141] In humans, a study conducted between children living in Burkino Faso who typically consume high-fiber diets, compared to Italian children who typically consume a Western Diet, found that the Western diet group had significantly lower richness and biodiversity than the high-fiber group.[25] Our study confirms these findings in an exclusively Hispanic population, adding that specifically saturated fat is linked to a less diverse microbiome.

A higher abundance of *Firmicutes* in the microbiome has been linked to unhealthy diets and metabolic profiles.[140] Multiple studies in mice comparing standard chow to mice put on a high fat diet consistently showed significantly decreased counts of *Bacteroidetes* and increased amounts of *Firmicutes* and *Proteobacteria*.[30,141] In humans, the study done between children living in Burkino Faso and Italy found diets higher in fat to have higher

counts of *Firmicutes*.[25] HMP is one of the few ongoing human studies in the United States that showed that a diet high in fat and low in dietary fiber was positively linked to phylum-level *Bacteroidetes* and *Actinobacteria* and inversely linked with *Firmicutes* and *Proteobacteria*.[74] On the phylum level, like HMP, the current study found types of *Firmicutes* (*Bacillales*) and *Proteobacteria* (*Campylobacterales*) to be significantly less abundant in the participants who exceeded saturated fat recommendations. Unlike HMP, this study did not find any other dietary variable, specifically dietary fiber, to be linked to the gut microbiome. It is important to note that there are several differences in methodology between the HMP and the current study, including the HMP used food-frequency questionnaires to assess dietary intake, ran Spearmen correlations, and did not report on the order-level of the bacteria, therefore the results are not directly comparable. However, the current findings support these studies done in mice and humans that diets high in saturated fat are linked to an altered microbiome.[30]

This study was the first to examine breakfast eating vs. breakfast skipping and meal frequency. Although decreased eating frequency has been linked to increased visceral adipose tissue, body fat, and obesity risk in this population,[16,142] eating frequency was not shown to affect the microbiome. Multiple studies have confirmed that a whole-grain breakfast contributes to a prebiotic effect to the human gut microbiota.[73,136,137] However, breakfast skipping which has been associated with visceral fat and insulin indices in overweight Latino youth,[15] was not significantly related to the gut microbiome. This suggests that in this cross-sectional study, nutrient content rather than nutrient timing contributed to the composition of the human gut microbiome. Longitudinal and

intervention studies are needed to assess the impact of nutrient timing on the gut microbiome.

We found increases in the relative abundance of microbiomes that have been associated with the breakdown of fiber. *Victivallales* has been detected in cows and aids in vegetal fiber degredation.[143] *Methanobacteriales* uses H_2 to reduce CO_2 to CH_4, which is produced exclusively through breaking down carbohydrates in humans. [144,145] When we examined the microbiomes of those who met fiber recommendations and those who fell below, there were no significant differences. However, subjects who exceeded fat intake versus those that met them had significantly lower intakes of dietary insoluble fiber (13.41 ±0.95 grams/day vs 10.74 ±0.74 grams/day; p=0.04). These findings suggest that diets high in saturated fat lack bacteria that break down fiber. This could possibly be due to bacteria that break down fat competing with bacteria that break down fiber, or that the population in our study had a fairly homogenous diet regarding fiber.

Two other bacteria found to be significantly less abundant in those who exceeded saturated fat recommendations were *Campylobacteria* and *Bacillales*. *Campylobacteria* is recognized as bacteria associated with diarrhea, however its role or proof of being a pathogen has not been proven. [132] *Bacillales* are known to produce antimicrobial compounds, which function as an antibacterial or antifungal and used to treat or prevent infection.[146] The specific functions of these bacteria in humans remain elusive and further examination would be important.

The limitations of this study include the cross-sectional design and the relatively small sample size. This population was homogenous in ethnicity, location, and age,

eliminating possible confounders. However, in some instances the diet was homogenous as well such as protein recommendations being met and fiber recommendations not being met by nearly the entire sample. A larger sample size, a more diverse diet, and deeper amplification are needed to further explore significant differences in specifying bacteria available in smaller amounts.

In summary, our study is consistent with other studies that have reported that subjects with diets high in saturated fat have a less diverse microbiome. Saturated fat has been routinely linked to cardiometabolic diseases[82,105,141] and a decrease in microbial diversity has been linked to metabolic diseases such as obesity and T2D.[24] The effect of saturated fat on the microbiome could be one mechanism underlying these diseases. Although diet may be playing a role in cardiometabolic disease etiology, it may be a small role and more exploration is needed to confirm the potential mechanism.

Few studies have implicated human consumption of fat on the overall decreased diversity of the microbiome and order-level identification of specific bacteria. Metabolic diseases disproportionally affect Hispanics[11] and no studies have examined the effects of diet on the microbiome in an exclusively Hispanic college population. This study suggests that a decrease in saturated fat intake is recommended in order to increase biodiversity and could have downstream impacts on decreases in cardiometabolic and/or inflammatory diseases. More longitudinal and intervention studies are warranted to understand the role that diet plays on the gut microbiome and subsequent disease risk.

Chapter 6: Conclusion

The purpose of this research was to study the relationship between diet, gut microbiome, and disease risk in a vulnerable and understudied population of Hispanic college freshmen. Specifically, this dissertation characterized the young adult Hispanic gut microbiome and examined the relationship between 1) diet and eating patterns with adiposity and metabolic measures, 2) adiposity and metabolic measures with the gut microbiome, and 3) the gut microbiome and diet and eating patterns. This cross-sectional study of college Hispanic freshmen (18-19 years of age) revealed that total dietary fat and saturated fat were positively linked to SAT, total body fat, insulin, insulin resistance, leptin, and CRP, and dietary saturated fat was linked to hepatic fat. Furthermore, the odds of having NAFLD increased by 34% for every percent increase of dietary saturated fat. Carbohydrates were positively linked to CRP, total sugar was positively linked to triglycerides, and added sugar was positively linked to VAT. On the other hand, fiber was inversely linked to hepatic fat, glucose, insulin, insulin resistance, and leptin. These results support existing literature that high intakes of dietary saturated fat and low intakes of dietary fiber are linked to obesity and related diseases.

Saturated fat was also the only significant variable linked to the gut microbiome. Those who met saturated fat recommendations had significantly higher biodiversity compared to those who exceeded saturated fat recommendations. Upon further analysis, those who exceeded saturated fat had decreased relative abundance of the potentially beneficial bacteria: *Victivallales, Methanobacteriales, Campylobacterales*, and *Bacillales*. *Victivallales* and *Methanobacteriales* participate in carbohydrate breakdown;

Campylobacterales have an unknown function; and *Bacillales* upregulates antibacterial and antifungal compounds. [132,143–145] However, when analyzing the gut microbiome in subjects with and without NAFLD, overall diversity was not significantly different. This suggests that the gut microbiome may not explain the link between saturated fat and NAFLD seen in Chapter 3.

When the gut microbiome was analyzed by tertiles of adiposity and metabolic measures, total percent body fat, insulin, and LDL were the only significant variables. Subjects with high percent body fat and insulin compared to those with low and moderate percent body fat and insulin had lower microbial composition diversity. On the contrary, participants with low and moderate LDL levels compared to subjects who had high levels of LDL had lower microbial composition diversity. Subjects with high percent body fat had lower relative abundance of *Methanobrevibacter, Akkermansia*, and *Clostridiales*. *Methanobrevibacter* and *Akkermansia* have been correlated with leanness, although exact function is unknown, and *Clostridiales* participates in degradation of fiber. Subjects with high insulin had lower relative abundance of *Proteobacteria*. However, no conclusions can be made without further specificity of the bacteria species within this very diverse phylum. Low values of LDL had decreased relative abundance of *Jonquetella anthropi, Actinomyces europaeus, Pyramidobacter, Peptococcus, Moryella, Mobilincus,Campylobacter, Facklamia, Mogibacterium, Gallicola, WAL_1855D,* and *I-68*, most of which do not have designated functions. *Jonquetella anthropic, Actinomyces europaeus*, and *Pyramidobacter* aid in the production of short chain fatty acids, which decrease inflammation. [61,125–128] *Peptococcus* and *Moryella* are involved in various forms

of transportation and signaling to aid in the function of LDL. [129,130] These results confirm that the gut bacteria that are present in leaner subjects participate in fiber digestion. However, the LDL findings were contrary to the hypothesis. Upon further inspection, the range of LDL values fell within the recommended range; meaning subjects who had lower amounts of LDL had insufficient stores of LDL compared to those who had optimal levels of LDL. In addition, the specific bacteria found in those who had optimal levels compared to those who had suboptimal levels were bacteria that aided in LDL function. This provides support that LDL is beneficial to the body at certain ranges and that the microbiome is a mechanism for blood lipids. Therefore, the gut microbiome may be a mediating factor involved in cardiovascular disease risk.

Although there were many significant results, there were also many null results. Eating patterns such as frequency of meals and breakfast did not achieve significance; suggesting nutrient content may be a stronger driver of the microbiome than nutrient timing. However, intervention and longitudinal studies are needed to assess the true impact of nutrient timing. Tertiles of dietary variables did not achieve significance in microbiome diversity between subjects, nor did adiposity and metabolic measures split into healthy versus unhealthy ranges. This is most likely due to the homogeneity of the sample, and in some instances the diet. Having a sample of the same age, ethnicity, and location limited confounders. However, this also inadvertently created bias. This group did not provide enough unhealthy subjects to analyze overall disease risk. These Hispanic college freshmen were leaner, more active, and more metabolically healthy than the general Hispanic adult population, although their diets were similar to the national

diet averages.[97,103] However, having a homogenous sample was ideal for characterizing a health emerging young adult Hispanic microbiome.

The young adult Hispanic microbiome was predominantly made up of the phylum *Firmicutes*, followed by *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Verrumicrobia*. Figure 5.2 shows how the gut microbiome of Hispanic youth matches up to healthy adult populations of differing ages (18 years of age and up) and ethnicities in both Europe (MetaHit) and the US (HMP). [69] The Hispanic microbiome is more similar to those subjects in Europe. In addition, when comparing the Hispanic microbiome to children (1-6 years of age) in Italy and in Burkino Faso, the young adult Hispanic microbiome is more similar to the Italian children than it is to the HMP, despite being a US population.[25] This suggests that the microbiome of college students may be more similar to a child microbiome than an adult microbiome and that age may be a more defining factor than location. Population wide samples should control for age and ethnicities when characterizing a healthy human gut microbiome.

In conclusion, this was the first study to examine the relationship between dietary intake, adiposity, metabolic parameters, and the gut microbiome in a Hispanic college population. It is also the first study to directly examine the relationship between diet, metabolic diseases, and the gut microbiome in an exclusive Hispanic population. Findings support previous studies that diets high in fiber compared to those low in fiber are linked to lower adiposity, healthier metabolic outcomes, and more diverse microbiomes, specifically bacteria that breakdown fiber. [49,81,143] On the other hand, diets high in saturated fat compared to those low in saturated fat have higher adiposity and

decreased microbiome biodiversity, specifically a reduction in metabolically beneficial bacteria[25,74,141] Saturated fat exhibited a strong relationship with the incidence of NAFLD, like that found in overweight and obese women in another study.[89] However, while the gut microbiome may still be a potential mechanism between saturated fat and NAFLD, in this study, the microbiome did not significantly modulate the relationship. This finding reinforced previous studies that reported no significant difference between subjects with and without NAFLD.[42,43] This could be due to the number of NAFLD subjects and should be studied in a larger sample and more directly. A saturated fat intervention in NAFLD patients would be a better design for determining the mechanism involving the microbiome on diet and disease. On the contrary, LDL was shown to have a relationship with the gut microbiome and HDL was approaching significance, attesting to the gut microbiome being the potential mechanism behind cardiovascular diseases.

These results collectively indicate that this population would benefit from an intervention targeting saturated fat. These participants were not sugar consumers as suspected with an average of 70.5 grams/day in comparison to the national average in a similar age group of 105 grams/day,[147] which suggests that tailored interventions for different populations are warranted. In this case, Hispanic college freshmen consumed high amounts of saturated fat, which correlated significantly with the microbiome. Comparing baseline microbiome bacteria diversity and abundance over time pre and post saturated fat intervention would explore a potential cause and effect relationship between saturated fat and microbiome diversity. A suggested intervention of one year or longer would be useful to illustrate any lasting effects. This intervention would be particularly

interesting in subjects with NAFLD in order to directly study any impacts being made in disease progression via diet and microbiome. In addition, despite the amounts of fiber being consumed below recommended amounts, there was a relationship between fiber, adiposity, and some metabolic measures. Further research on a range of fiber intake, as well as a possible dietary intervention replacing saturated fat with fiber, would be beneficial for studying dietary mechanisms and disease prevention.

Decreases in microbial biodiversity are linked to metabolic disease states that plague the Hispanic population such as obesity, diabetes, NAFLD, and cardiovascular disease. This study highlights that diet is linked to the gut microbiome and may be a potential mechanism explaining how diet impacts obesity and its accompanying morbidities. These findings support other studies that show that diets high in dietary fiber and low in saturated fat are linked to low levels of adiposity, metabolic disease risk, and healthier microbiome profiles as predicted in the hypothesis. More longitudinal and intervention studies are needed to better understand these relationships.

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